理學博士學位請求論文

일반 PTS 단백질 HPr에 의한 포도당과 만니톨 사이의 당 선호도 조절

Determination of glucose preference over mannitol by a general PTS component HPr

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生命科學部

崔 滿 珪

Determination of glucose preference over mannitol by a general PTS component HPr

by

Mangyu Choe

Under the supervision of

Professor Yeong-Jae Seok, Ph. D

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School of Biological Sciences

Seoul National University

ABSTRACT

Mangyu Choe

School of Biological Sciences

The Graduate School

Seoul National University

Bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS) plays a major role in the transport of a variety of sugar substrates and phosphorylates them at the same time. It consists of two general cytoplasmic proteins, enzyme I (EI) and the histidine phosphocarrier protein, HPr, which are used for all PTS sugars, and a variable number of sugar-specific transport complexes collectively known as enzyme II (EII). EII complexes usually consist of three domains (A, B and C). They contain a membrane-bound C domain that takes charge of transporting sugar into the cytoplasm. These PTS proteins are known to participate in intracellular signal transduction and regulation of metabolic enzymes as well as transport function.

Carbon catabolite repression (CCR) is the regulatory phenomenon to ensure sequential utilization of carbon sources in microorganisms. Its molecular mechanism has been most intensively studied in the model organisms *Escherichia coli* and *Bacillus subtilis*. Glucose is the most preferred carbon source in these organisms, being transported via the PTS. Previous studies on CCR suggested a model based on 'inducer exclusion' that includes inhibition of transport of several non-PTS sugars and 'induction

prevention' that includes lowering of adenylate cyclase activity. Both pathways are regulated mainly by the phosphorylation state of enzyme IIA^{Glc} ($EIIA^{Glc}$) encoded by the *crr* (catabolite repression resistant) gene. In *E. coli*, the preferential utilization of glucose over non-PTS carbon sources such as lactose is known to be strictly dependent on the phosphorylation state of the $EIIA^{Glc}$. On the contrary, the mechanism of the preference among PTS sugars has never been addressed. The regulatory mechanism of glucose-mannitol diauxie is thought to be far different from that of glucose-lactose diauxie.

In this study, the molecular mechanism of the glucose preference over another PTS sugar, mannitol in *E. coli* was investigated. Surprisingly, the *crr* mutant retained the glucose preference over mannitol, whereas this selectivity disappeared in a mutant devoid of MtlR that negatively regulates regulating the expression of the *mtlADR* operon required for the transport and metabolism of mannitol. MtlR showed a high affinity interaction with the dephosphorylated form of the general PTS component HPr, which increased during glucose uptake. This interaction decreased the expression of the *mtlADR* operon by enhancing the repressor activity of MtlR and resulted in the inhibition of mannitol utilization. The regulatory machinery of the *mtl* operon mediated by MtlR is still unclear since MtlR cannot directly interact with the promoter region of the *mtl* operon. To gain further insight, the crystal structure of MtlR-HPr complex was solved but no DNA binding domain was identified. Based on various experiments performed in this study, an unknown DNA-binding protein seems to be required to mediate the DNA binding of MtlR. Taken together, the results reveal HPr as the key

player in the sugar selectivity between glucose and mannitol.

Key words :

Carbon catabolite repression, glucose-mannitol diauxie, protein-protein interaction, PTS

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ABBREVIATIONS

E. coli, *Escherichia coli*

CCR, carbon catabolite repression

PTS, phosphoenolpyruvate:sugar phosphotransferase system

PEP, phosphoenolpyruvate

AC, adenylate cyclase

EI, enzyme I of the PTS

HPr, histidine phosphocarrier protein

$EIIA^{Glc}$, the glucose specific enzyme IIA of the PTS

mtl operon, mannitol operon

Glc, glucose

Mtl, mannitol

NAG, N-acetyl-D-glucosamine

CRP, cyclic AMP receptor protein

cAMP, cyclic AMP

MALDI-TOF, matrix-associated laser desorption ionization time-of-flight

SDS, sodium dodecyl sulfate

PAGE, polyacrylamide gel electrophoresis

A_{600}, optical density at 600 nm

EMSA, Electrophoretic Mobility Shift Assay

SEC-MALS, Size Exclusion Chromatography with Multi-Angle Light Scattering

Chapter I

Introduction

1. Carbon catabolite repression

1.1. Overview of carbon catabolite repression

The preferential utilization of a certain carbon source over others has been observed in various model organisms. Carbon catabolite repression (CCR) is generally regarded as the regulatory mechanism to ensure sequential utilization of carbohydrates in microorganisms (Loomis *et al.*, 1967). The presence of a carbon source rapidly metabolizable, such as glucose, in the culture medium inhibits the gene expression and/or protein activity involved in the metabolism of secondary carbon sources. Sugars transported by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) are generally believed to be preferred over non-PTS carbon sources in various bacterial species (Deutscher *et al.*, 2006; Görke *et al.*, 2008).

Molecular mechanism for inhibition of expression and/or activity of proteins required for the transport and metabolism of less-preferred carbon sources in the presence of a preferred carbon source has been most extensively studied in *E. coli* and the best-studied example of this CCR is the glucose-lactose diauxie, which was first observed by Jacques Monod in 1942 (Monod, 1942). If *E. coli* grows in a medium containing both glucose and lactose, it uses glucose preferentially over lactose until glucose is depleted. Then after a short lag period, growth resumes with lactose as the carbon source. The biphasic growth pattern shown in two carbon sources is called diauxic growth. Extensive studies in CCR provides a regulatory model in *E. coli*. The current model for the glucose preference over other sugars involves inducer exclusion and induction

prevention (Görke *et al.,* 2008), both of which are strictly dependent on the phosphorylation state of $EIIA^{Glc}$ (also called Crr for catabolite repression resistant) in *E. coli* (Fig. 1).

1.2. Inducer exclusion

According to previous studies, it is already known that dephospho-$EIIA^{Glc}$ can interact with and inhibit several non-PTS permeases such as lactose permease and melibiose permease (Postma *et al*., 1993). In addition, it can interact with and inhibit glycerol kinase. Therefore, the transport and/or metabolism of less preferred carbon sources, such as lactose, is prevented in the presence of glucose because $EIIA^{Glc}$ is dephosphorylated during the glucose consumption (Fig. 1). This phenomenon is called inducer exclusion, since the preferred substrate or its metabolic derivatives inhibits the entry of molecules that induce other metabolic systems (Postma *et al.*, 1993).

1.3. Induction prevention

After glucose is exhausted in culture medium, $EIIA^{Glc}$ is phosphorylated and phospho-$EIIA^{Glc}$ then stimulates adenylyl cyclase, an enzyme converting ATP into cyclic AMP (cAMP) (Park *et al.,* 2006) (Fig. 1). cAMP plays a central role in gene expression in enteric bacteria. Together with cAMP receptor protein CRP, it functions as a transcriptional activator involved in the global regulation of expression of numerous genes and operons required for the transport and/or metabolism of less preferred carbon sources. Consequently, the synthesis of cAMP required for the induction of these genes

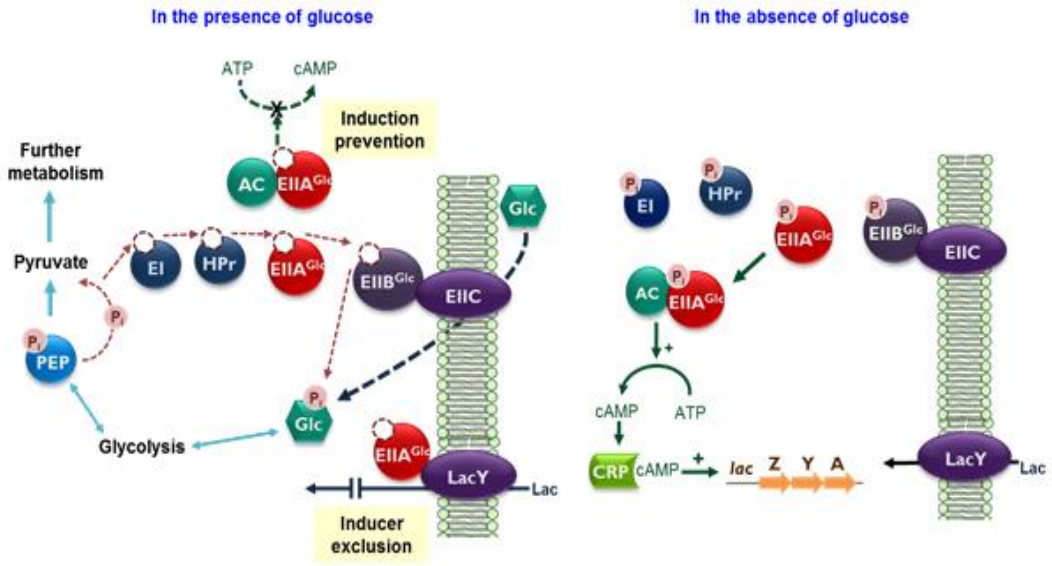

Figure 1. A schematic view of the current model for carbon catabolite repression (CCR) in *E. coli*. Glucose preference over non-PTS carbon compounds is currently explained by inducer exclusion and induction prevention in *E. coli*. Because dephospho-EIIA^{Glc} interacts with and inhibits several non-PTS permeases including the lactose permease (LacY), the transport of less preferred carbon sources is prevented in the presence of glucose. This is called "inducer exclusion." Dephospho-EIIA^{Glc} cannot stimulate AC, the synthesis of cAMP required for the induction of these genes and operon is prevented in the presence of glucose. This is termed "induction prevention."

and operons is prevented during the growth on glucose. This is termed induction prevention. Based on inducer exclusion and induction prevention, $EIIA^{Glc}$ has been regarded as the central processing unit of CCR in *E. coli* and other enteric bacteria (Deutscher *et al.,* 2006).

2. Phosphoenolpyruvate:sugar phosphotransferase system (PTS) in *Escherichia coli*

2.1. Overviews of PTS

Among the diverse transport mechanisms for the uptake of carbon sources in bacteria, the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) plays the major role in the transport of a variety of carbon sources (Postma *et al.,* 1993). It is a multicomponent system that consists of two general components, enzyme I (EI) and the histidine-containing phosphocarrier protein (HPr), which are common to all PTS sugars, and a variable number of sugar-specific transport complexes collectively known as enzyme II (Postma *et al.,* 1993). Each EII complex usually has three domains, two cytosolic domains (EIIA and EIIB) and one membranous domain forming the sugar translocation channel (EIIC) (Deutscher *et al.,* 2014). For example, the *E. coli* glucose-specific EII consists of $EIIA^{Glc}$ encoded by the *crr* gene and $EIICB^{Glc}$, where the cytosolic EIIB domain is fused to the carboxy-terminal end of EIIC in a single polypeptide encoded by the *ptsG* gene. EI transfers a phosphoryl group from PEP to HPr, and HPr then sequentially transfers the phosphoryl group through the various

EIIAs and EIIBs to the incoming sugars. In short, the PTS catalyzes the concomitant phosphorylation and translocation of many sugars across the cytoplasmic membrane (Deutscher *et al.*, 2006).

PTS is ubiquitous in eubacteria but do not occur in archaebacteria and eukaryotes. The general PTS components, EI and HPr, are highly conserved in all bacteria whereas the number and structure of sugar-specific PTS transporter vary between species. PTS transporters can be grouped into four structurally different families by sequence comparison (Postma *et al.*, 1993) (Fig. 2). The *E. coli* genome encodes 38 different PTS proteins, 33 of which are subunits belonging to 22 different enzyme II components (Tchieu *et al.*, 2001).

PTS proteins play diverse roles in intracellular signal transduction in addition to their transport function. These proteins regulate targets either by protein-protein interaction or by phosphorylation. Unphosphorylated EI inhibits CheA, the sensor kinase of the bacterial chemotaxis machinery (Lux *et al.*, 1995) and dephosphorylated HPr stimulates the activity of glycogen phosphorylase (Seok *et al*., 1997). Dephosphorylated $EIIA^{Glc}$ inhibits glycerol kinase (Hurley *et al*., 1993), and regulates FrsA (Koo *et al*., 2004), and phospho-$EIIA^{Glc}$ activates adenylate cyclase (Park *et al*., 2006).

2.2. General and sugar-specific components of PTS

2.2.1. General components of PTS

Two general components of PTS are encoded in the *ptsHIcrr* operon. *crr*, the gene for $EIIA^{Glc}$, encoded in the *ptsHIcrr* operon with general components appears the

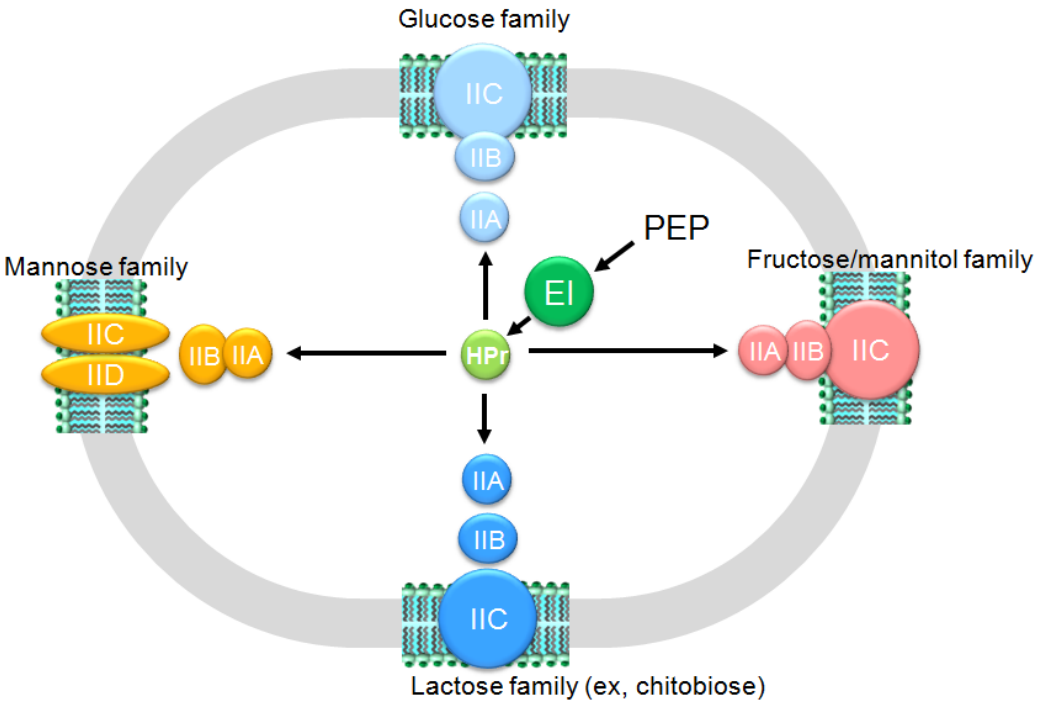

Figure 2. Phosphoenolpyruvate:sugar phosphotransferase system (PTS) in *E. coli*. PTS consists of Enzyme I and HPr which are common to all PTS sugars and sugar-specific Enzyme II. It transports and phosphorylates a variety of sugars. The phosphoryl group is transferred from PEP to final sugar sequentially as follows; PEP → EI → HPr → sugar-specific EII → sugar.

importance of allosteric regulatory functions of $EIIA^{Glc}$ on carbon source utilization. The *ptsHIcrr* operon is induced by growth on glucose.

Enzyme I (EI) is encoded by the *ptsI* gene and its molecular weight is 63.5 kDa. It transfers phosphoryl group from PEP to HPr and consist of two domains; the N-terminal domain which functions in the phosphorylation of HPr and C-terminal domain which is important in PEP binding and dimerization. Divalent cations such as Mg^{2+} or Mn^{2+} and dimeric form of EI are required for the autophosphorylation of EI (Chauvin *et al.,* 1994; Seok *et al.,* 1998).

Histidine-containing phosphocarrier protein (HPr) is a small monomeric thermostable protein with molecular weight of 9.2 kDa (De Reuse *et al.,* 1985). These properties allow its purification from a variety of organisms. Based on its simplicity to purification, extensive studies were done in various organisms and it is the first PTS protein whose three-dimensional structure has been reported by X-ray crystallography and NMR (Wittekind *et al.,* 1990; Herzberg *et al.,* 1992). It functions in transferring the phosphoryl group from EI and to the various sugar specific enzyme IIs. In addition to its transferring activity, HPr is involved in several regulatory mechanisms. HPr stimulates the activity of glycogen phosphorylase when it is dephosphorylated (Seok *et al.,* 1997). Recently, it has been reported that dephosphorylated HPr interact with Rsd, anti-sigma factor of sigma D (Park *et al.,* 2013).

Enzyme IIs (EIIs) are the sugar-specific component in PTS (Fig. 2). They generally consist of three domains (A, B and C) whose composition is various; the domains of

EIIs are separated from or fused to each other in a variety of ways. For example, EII^{Glc} consists of cytosolic $EIIA^{Glc}$ and membrane-bound $EIICB^{Glc}$ whereas both A and B domains are fused in a single soluble peptide and two intergral membrane proteins (IIC^{Man} and IID^{Man}) are involved in mannose translocation in the case of mannose PTS. In the case of cellobiose PTS in *E. coli*, each functional domain of its EII is expressed as a separate protein. Two soluble proteins (IIA^{Cel} and IIB^{Cel}) that each contains a site of covalent phosphorylation, and one membrane-bound protein (IIC^{Cel}) form the cellobiose PTS. Regardless of the diversity of EIIs, all kinds of EIIA and EIIB take part in the phosphoryl group transfer from PEP to the incoming diversity of EIIs, all kinds of EIIA and EIIB take part in the phosphoryl group transfer from PEP to the incoming carbohydrates. The membranous domain (usually EIIC) forms the translocation channel and the part of specific substrate binding sites.

2.2.2. Glucose PTS

Four proteins required for glucose transport by PTS, EI, HPr, $EIIA^{Glc}$ and $EIICB^{Glc}$, are encoded by the *ptsHIcrr* operon and *ptsG* gene. The proximity of the *crr* gene to those central PTS proteins appears to reflect the importance of the regulatory functions of $EIIA^{Glc}$ on carbon source metabolism. Both *ptsHIcrr* and *ptsG* operons are induced by growth on glucose.

$EIIA^{Glc}$ (18.2 kDa) of *E. coli* is phosphorylated by P-HPr at His-90 residue as an intermediate in PEP-dependent phosphotransfer to incoming glucose (Dorschug *et al.*, 1984; Presper *et al.*, 1989). The N-terminus of $EIIA^{Glc}$ appears to be important for its

interaction and phosphorylation of $EIICB^{Glc}$ based on the proteolytic cleavage of the first 7 amino-terminal residues decreased the rate of phosphotransfer from $EIIA^{Glc}$ to $EIICB^{Glc}$ compared to intact $EIIA^{Glc}$. Site-directed mutagenesis studies coupled with the recent three-dimensional structure of $EIIA^{Glc}$ have provided clues to structure-function relationships in the phosphotransfer activity of $EIIA^{Glc}$. As expected, mutation of the His-90 in $EIIA^{Glc}$ from *E. coli* with glutamine (Q) resulted in unphosphorylatable protein that could not accept phosphoryl group from P-HPr. If the same replacement was made in the His-75 of $EIIA^{Glc}$, the mutant protein could still be phosphorylated by P-HPr but could not transfer phosphoryl group to $EIIBC^{Glc}$ (Presper *et al.*, 1989).

The *E. coli* EIICB protein, whose molecular weight is 50.7 kDa, consists of two domains; the N-terminal membrane-bound domain (EIIC; 41.1 kDa) which determines the substrate selectivity, and the C-terminal cytoplasmic domain (EIIB; 9.6 kDa) which is phosphorylated at Cys-421 by $P\text{-}EIIA^{Glc}$. EIIBC normally recognizes glucose as well as methyl-α-D-glucoside, 5-thio-D-glucoside, L-sorbose, mannose and 2-deoxyglucose with a low affinity (Garcia-Alles *et al*., 2002). Arg-424 and Arg-426, next to Cys-421, are crucial for the phosphorylation and transport of glucose, but not for the phosphorylation $EIIBC^{Glc}$ by $P\text{-}EIIA^{Glc}$. Mutant protein with Cys-421 to serine (C421S) only abolishes phosphorylation of $EIIBC^{Glc}$ by $P\text{-}EIIA^{Glc}$. $EIIBC^{Glc}$ comprises eight putative membrane spanning segments, a topology of which was first derived from the activity of fusion proteins between $EIICB^{Glc}$ and β-galactosidase or alkaline phosphatase and further confirmed by random linker insertion mutagenesis. A projection map of 2D crystals indicates a dimeric structure of $EIICB^{Glc}$, in agreement with results of inner-

allelic complementation crosslinking and co-purification of heterodimers (Siebold *et al.*, 2001).

2.2.3. Mannitol PTS

Manntiol transporter is encoded by the *mtlA* gene which is transcribed with *mtlD* and *mtlR* as a mannitol (*mtlADR*) operon. The *mtlADR* operon encodes mannitol PTS transporter ($EIICBA^{Mtl}$), mannitol-1-phosphate 5-dehydrogenase, converting mannitol-1-phosphate to fructose-6-phosphate in a reversible reaction (Figge *et al.*, 1994), and the transcriptional regulator of the mannitol operon, whose function is still largely unknown.

MtlA belongs to the functional superfamily of the PTS and transports the exogenous mannitol into the cytoplasm as a phosphate ester, mannitol-1-phosphate. The transport of mannitol is simultaneous with phosphorylation of mannitol, like other PTS (Postma *et al.*, 1993). MtlA ($EIICBA^{Mtl}$) possesses three domains in a single polypeptide chain with the domain order IIC-IIB-IIA. IIC is a N-terminal hydrophobic domain that resides in the inner membrane and IIBA is a C-terminal hydrophilic domain that is located on the cytoplasmic side of the membrane (Lee *et al.*, 1983). The N-terminal domain of MtlA contains 6 transmembrane α-helical segments and is important for binding and translocation of mannitol (Lolkema *et al.*, 1990). His-554 and Cys-384 located in the hydrophilic domain are responsible for the phosphotransfer activity of MtlA. His-554 accepts a phosphate group from cytoplasmic P-HPr and Cys-384 is the site required for phosphotransfer to D-mannitol. MtlA functions as a homodimer and it contains one high

affinity mannitol binding site and one low affinity binding site (Pas *et al.,* 1988). The mannitol binding site is located in the middle of the membrane and mannitol is phosphorylated at the same site.

MtlR is a transcriptional repressor of mannitol operon which controls the transport and catabolism of the mannitol. It is known as a DNA-binding transcriptional repressor but no consensus sequence has been identified (Figge *et al.,* 1994). The crystal structure of MtlR from *Vibrio parahaemolyticus* and its homolog, a YggD protein from *Shigella flexneri*, have been determined but the regulatory mechanism of MtlR is still unclear (Tan *et al.,* 2009).

The mannitol operon is inducible in the presence of mannitol by 20 fold. The expression of operon is positively controlled by cAMP-CRP complex and negatively by the catabolite repressor/activator (Cra) protein. The MtlR protein is a negative transcriptional regulator of the *mtlADR* operon and there are several palindrome sequences within the promoter region and the *mtlR* gene that may work to regulate transcription or translation (Figge *et al.,* 1994).

3. PTS-mediated regulation

3.1. Interaction between acetate kinase and EI

Acetate kinase catalyzes the conversion of acetate and ATP into acetyl phosphate and inorganic phosphate (Pi). During this reaction, a phosphate group linked to the enzyme via an acyl phosphate, a high-energy bond. In the presence of EI, HPr and $EIIA^{Glc}$,

acetate could be phosphorylated by ATP via acetate kinase (Fox *et al.*, 1986). Phosphorylated EI can directly transfer its phosphoryl group to acetate kinase, and the phosphate group of P-EIIA^{Glc} can be transferred to acetate kinase in the presence of EI and HPr. This reaction could form a metabolic link between the PTS and the enzymes involved in the Krebs cycle (Fox *et al.*, 1986).

3.2. Interaction between HPr and glycogen phosphorylase

Glycogen phosphorylase catalyzes the breakdown of glycogen into glucose-1-phosphate while the PTS affects the uptake of sugar substrates, such as glucose, and hence both play a central role in carbohydrate metabolism. Direct interaction between HPr and glycogen phosphorylase were found by surface plasmon resonance (Seok *et al.*, 1997). Although both HPr and P-HPr were shown to interact with glycogen phosphorylase, only dephosphorylated HPr activated the enzymatic activity of glycogen phosphorylase. Moreover, it was shown that HPr regulates the intracellular concentration of glycogen *in vivo*, and thus, crosstalk between these two important carbon metabolic pathways (glucose and glycogen) are mediated by the phosphorylation state of HPr of the *E. coli* PTS. Furthermore, it was shown that the amount of unphosphorylated HPr but not of glycogen phosphorylase is critical for the regulation of the intracellular glycogen level. Because the phosphocarrier function of HPr in the PTS allows the protein to exist in both phospho- and dephospho- forms, glycogen could be degraded in the presence of glucose (Koo and Seok, 2001).

3.3. Regulation of FrsA by EIIA^{Glc}

A novel EIIA^{Glc}-binding protein from *E. coli* extracts was discovered by Koo *et al*., (2004) using protein ligand-fishing asssay and interaction between two proteins were confirmed by surface plasmon resonance. This protein, which named FrsA (fermentation/respiration switch protein), is the 47-kDa product of the *yafA* gene, previously denoted as "unknown function." FrsA forms a 1:1 heterodimer complex specifically with the dephosphorylated form of EIIA^{Glc}, with the highest affinity of any protein thus far shown to interact with EIIA^{Glc}. Homology search of FrsA indicated that orthologs of FrsA exist only in facultative anaerobes belonging to the gamma-proteobacterial group. Increased expression level of FrsA resulted in an elevated fermentation rate on several sugars including glucose with the concomitant accumulation of mixed-acid fermentation products, while disruption of *frsA* increased cellular respiration on these sugars. Based on these results, EIIA^{Glc} regulates the flux between respiration and fermentation pathways by sensing the available carbon sources via a phosphorylation state-dependent interaction with FrsA.

3.4. Interaction between adenylate cyclase and EIIA^{Glc}

In *E. coli*, cAMP is produced by adenylate cyclase (AC) which is encoded by the *cyaA* gene. AC is a monomer of molecular weight of 97,856 and catalyzes the synthesis of cAMP by an intramolecular transfer of the phosphate of ATP to 3'-hydroxyl group, releasing pyrophosphate. This protein is soluble or loosely membrane bound and unstable during the purification process (Yang and Epstein, 1983). The enzyme activity

of AC is very low, with turnover number of about 100 min^{-1}. AC is composed of two domains, an amino-terminal catalytic domain and a carboxy-terminal regulatory domain (Roy *et al.*, 1983). The carboxy-terminal domain is required for glucose-specific regulation of AC.

It has been proposed that both dephosphorylated and phosphorylated form of $EIIA^{Glc}$ interacts with AC. However, experiments using membrane-bound Tsr-AC showed that carboxy-terminal domain of AC specifically interacts with $EIIA^{Glc}$ and only the phosphorylated form of $EIIA^{Glc}$ stimulates AC activity (Park *et al.*, 2006).

4. Sugar preference between glucose and other PTS sugars

The current proposed model for glucose preference over other sugars which involves inducer exclusion and induction prevention, may explain preferred utilization of some PTS carbohydrates over non-PTS carbohydrates. However, preferential utilization among PTS sugars has also been observed several times, but the cause and mechanism of this hierarchical utilization remained unclear (Postma *et al.*, 1993) and cannot be explained by inducer exclusion nor by induction prevention.

5. The aims of this study

Like glucose, mannitol has also been perceived as a preferred PTS carbon source over some other PTS sugars such as sorbitol, galactitol and fructose in *E. coli* (Monod, 1942; Lengeler and Lin, 1972). Although no diauxie was observed when cells were provided

simultaneously with glucose and mannitol, the activity of the mannitol transport system was shown to be inhibited in the presence of glucose in *E. coli* (Lengeler and Steinberger, 1978). Such hierarchical utilizations among PTS sugars have been observed in various Gram-negative and in Gram-positive bacteria as well (Postma *et al.*, 1993). While competition among various EIIs for phospho-HPr has been suggested as a possible mechanism of the preference among PTS sugars (Postma *et al.*, 1993), this possibility has not been experimentally addressed. Therefore, in this study, the molecular mechanism of the preference between glucose and mannitol in *E. coli* was investigated.

Chapter II

Materials and Methods

1. Strains and plasmids

The strains, plasmids used in this study are listed in Table 1. Genomic DNA of *E. coli* MG1655, wild-type K-12 strain, was used as the template DNA for cloning. *E. coli* GI698 (La Vallie *et al.*, 1993) harboring the gene for the *c*I repressor under control of the promoter, and ER2566 (NEB) were used for overproduction of recombinant proteins. *E. coli* MG1655/pKD46 (Datsenko *et al.*, 2000) was used for disruption of chromosomal genes. An expression vector pRE1 (Reddy *et al.*, 1989), pET43.1a (Novagen), pACYC184 (Chang *et al.*, 1978), pBR322 (Bolivar *et al.*, 1977), which carries different antibiotic resistance markers and promoter of different strength, were used to construct overexpression vector of proteins.

2. Media and cell culture

LB medium (1% tryptone, 0.5% NaCl and 0.5% yeast extracts) and synthetic minimal medium (M9 salts, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and sugar) were used for routine bacterial culture. Synthetic minimal media supplied with various sugars were used for determination of bacterial growth curve and sugar preference test. The antibiotics ampicillin (100 μg/ml), kanamycin (20 μg/ml), chloramphenicol (20 μg/ml), and tetracycline (20 μg/ml) were added when required. For the overproduction of proteins using GI698 and the pRE1-based vector system, cells were grown in Rich medium (M9 salts, 1% casamino acid, 1% glycerol, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) at 30°C. Tryptophan of final concentration to be 100 μg/ml was added to the culture

Table 1. ***Escherichia coli*** **strains and plasmids used in this study.**

Strains or plasmid	Genotype or phenotype	Source or Reference
Strains		
MG1655	Wild-type *E. coli* K-12	(Blattner *et al*, 1997)
MG1655Δ*ptsH*	MG1655 *ptsH*::Km^r	(Park *et al*, 2013)
MG1655Δ*crr*	MG1655 *crr*::Tet^r	Lab stock
MG1655Δ*mtlR*	MG1655 *mtlR*::Km^r	This study
MG1655Δ*mtlA*	MG1655 *mtlA*::Cat^r	This study
MG1655Δ*mtlD*	MG1655 *mtlD*::Cat^r	This study
GI698	F$^-$ λ$^-$ *lacI*^{q} *lacPL8 ampC*::P_{tac} cI	(LaVallie *et al*, 1993)
GI698Δ*pts*	GI698 *ptsHIcrr*::Km^r	(Nosworthy *et al*, 1998)
ER2566	F- λ- *fhuA2* [*lon*] *ompT lacZ::T7 gene 1 gal sulA11* Δ(*mcrC-mrr*)*114::IS10 R*(*mcr-73::miniTn10-TetS*)*2 R*(*zgb-210::Tn10*)(*TetS*) *endA1* [*dcm*]	New England Biolabs
Plasmid		
pRE1	Expression vector under control of λP_L promoter, Amp^r	(Reddy *et al*, 1989)
pRE-HIcrr	pRE1-based expression vector for HPr, EI and EIIA^{Glc}	Lab stock
pRE-H(K27E)Icrr	Lys27 of HPr mutated to Glu in pRE-HIcrr	Lab stock
pRE-H(D69E)Icrr	Asp69 of HPr mutated to Glu in pRE-HIcrr	Lab stock
pSP100	pRE1-based expression vector for HPr	(Garrett *et al*, 1997)
pSP100 (R17A)	Arg17 of HPr mutated to Ala in pSP100	(Park *et al*, 2015)
pSP100 (Q51A)	Gln51 of HPr mutated to Ala in pSP100	(Park *et al*, 2015)
pSP100 (L55A)	Leu55 of HPr mutated to Ala in pSP100	(Park *et al*, 2015)
pSP100 (N12A)	Asn12 of HPr mutated to Ala in pSP100	(Park *et al*, 2015)
pSP100 (L47A/F48A)	Leu47 and Phe48 of HPr mutated to Ala in pSP100	(Park *et al*, 2015)
pACYC184	Cloning vector; Cm^r Tet^r	(Chang & Cohen, 1978)
pACYC-HPr	*E. coli ptsH* ORF and its promoter cloned between SphI and SalI sites of pACYC-184	Lab stock
pACYC-HPr(H15A)	His15 of HPr mutated to Ala in pACYC-HPr	Lab stock
pACYC-HPr(K27E)	Lys27 of HPr mutated to Glu in pACYC-HPr	This study
pACYC-HPr(L47A/F48A)	Leu47 and Phe48 of HPr mutated to Ala in pACYC-HPr	This study
pET43.1a	Cloning vector; Amp^r	Novagen
pET-MtlR	*E. coli mtlR* ORF cloned between NdeI and XhoI sites of pET43.1a	This study
pET-HisMtlR	His_6 tag added to the N-terminus of MtlR in pET-MtlR	This study
pET-HisMtlA (cyto)	His6 tag added to the cytosolic domain (AB) of *E.coli* MtlA cloned between NdeI and XhoI sites of pET43.1a	This study
pBR322	Cloning vector; Amp^r Tet^r	(Bolivar *et al*, 1977)
pBR322-MtlA	*E. coli mtlA* ORF with constitutive *CAT* promoter cloned between BamHI and SalI sites of pBR322	This study
pBR322-MtlD	*E. coli mtlD* ORF with constitutive *CAT* promoter cloned between BamHI and SalI sites of pBR322	This study
pBR322-MtlAD	*E. coli mtlAD* ORF with constitutive *CAT* promoter cloned between BamHI and SalI sites of pBR322	This study
pKD46	Red recombinase expression plasmid under the control of arabinose inducible promoter	(Datsenko & Wanner, 2000)

medium when the culture reached A_{600} of 0.4, and the cells were harvested 18-20 hrs after induction. For the overproduction of proteins using ER2566 and pET43.1a based vector system, cells were grown in LB medium at 37°C, and 1 mM of IPTG was added to the culture medium when the culture reached A_{600} of 0.5, and the cells were harvested 3-4 hrs after induction.

3. Protein purification

3.1. PTS proteins

E. coli GI698 transformed with pRE-*ptsHIcrr* was used for overproduction of the soluble PTS proteins (EI, HPr and $EIIA^{Glc}$), and these proteins were purified using ion-exchange and gel filtration chromatographies as described previously (Seok *et al.*, 1996).

3.2. His_6-tagged proteins

E. coli GI698*⊿pts* transformed with pRE-His-*ptsI*, pRE-His-*ptsH*, ER2566 transformed with pET-His-*mtlA*, pET-His-*mtlR* were used for overproduction of His6-tagged proteins. His-tagged proteins were purified using Clontech Talon metal affinity resin (Clontech Laboratoies, Inc.) according to the manufacturer's instructions. The cell pellet containing overexpressed His_6-tagged proteins were resuspended in binding buffer (20 mM HEPES-NaOH pH 7.4, 100 mM NaCl), disrupted by two passages through French press cell at 10,000 psi. After centrifugation at 9,300 × g for 20 min to

remove cell debris, the soluble fraction was mixed with Talon™ metal affinity resin, and the mixture was loaded onto Poly-Prep chromatography column (8 × 40 mm; Bio-Rad). The column was washed three times with wash buffer (10 mM imidazole added to binding buffer) and the bound to the resin were eluted with elution buffer (150 mM imidazole added to binding buffer). The fractions containing His_6-tagged proteins were concentrated using Amicon Ultracel-3K centrifugal filters (Millipore Ireland). To remove imidazole, the concentrated proteins was chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column (GE healthcare Life Sciences) equilibrated with buffer A (20 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 0.05% β-mercaptoethanol, 5% glycerol).

3.3. Non-tagged proteins

Non-tagged proteins were purified using MonoQ™ 10/100 GL (GE healthcare Life Sciences). HPr- or MtlR-overexpressed cell were resuspended in buffer B (20 mM Tris-HCl, pH 8.0, 50 mM NaCl) and distrupted by two passages through French press cell at 10,000 psi. After centrifugation at 100,000 × g for 60 min at 4°C, the supernatant was chromatographed on a MonoQ™ 10/100 GL and proteins were eluted with buffer C (20 mM Tris-HCl, pH 8.0, 1 M NaCl). The fractions containing HPr and MtlR were concentrated and chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column (GE healthcare Life Sciences) equilibrated with buffer A.

4. Direct interaction between MtlR and HPr

4.1. Ligand-fishing experiment using metal affinity chromatography

E. coli MG1655 or its isogenic *ptsH* mutant cells grown overnight in 500 ml of LB medium were harvested and resuspended in 10 ml of buffer A. Cells were distrupted by French press as described above. After centrifugation at 9,300 × g for 25 min at 4°C, the supernatant was divided into aliquots and mixed with a His-tagged protein as bait or buffer A as control. Each mixture was incubated with Clontech Talon metal affinity resin in 1.5 ml tube at 4°C for 30 min. After two brief wash with buffer A, the proteins bound to the Talon resin were eluted with 2X SDS loading buffer. 10 μl of each eluted samples were analyzed by SDS/PAGE, followed by staining with Coomassie brilliant blue R. A 9 kDa protein band eluted with as a significant amount when His_6-MtlR added crude extract from MG1655 were used, not MG1655⊿*ptsH*. In-gel digestion was carried out as described (Jeong *et al*., 2004). The peptide digested by trypsin were eluted three times with 20 μl of 5% trifluoroacetic acid containing 95% acetonitrile and concentrated to ~10 μl in vacuum centrifuge. MALDI-TOF mass spectrum was acquired using a Voyager-DE STR (Applied Biosystems Inc.). MALDI-TOF mass spectrometer equipped with a nitrogen laser (337 nM).

4.2. Co-purification of His_6-HPr with MtlR

To confirm the interaction between MtlR and HPr, co-purification experiment using metal affinity resin was performed. *E. coli* ER2566 transformed with pET-*mtlR* was used for overproduction of MtlR. Cell culture was harvested after 4 hr IPTG induction

and resuspended in binding buffer (20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl). Cells were disrupted by two passage through a French press at 10,000 psi, and centrifuged at 9,300 × g for 20 min to remove cell debris. After centrifugation, soluble fraction was mixed with various amount of purified His_6-HPr or same volume of binding buffer with or without 2 mM PEP and the mixtures were incubated with 50 μl of Talon™ metal affinity resin (Clontech). After 30 min incubation at 4°C, resin was washed twice with wash buffer (10 mM imidazole added to binding buffer). Proteins bound to the resin were eluted with elution buffer (150 mM imidazole added to binding buffer) and eluted protein samples were analyzed by SDS-PAGE with 16% polyacrylamide gel followed by Coomassie Blue staining.

4.3. Gel filtration chromatography

Size exclusion chromatography was performed on a Superose 12 10/300 GL column (GE Healthcare) using AKTA-FPLC system. Before injection, the column was preequilibrated with buffer A (20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, 0.05% β-mercaptoethanol, 5% glycerol). For each run, a 200 μl protein sample was injected into the column. Filtration was performed at room temperature at a flow rate of 1 ml/min. Chromatograms were recorded at 280 nm. Each fractions were analyzed by SDS-PAGE with 16% polyacrylamide gel followed staining with Coomassie Blue.

4.4. Surface plasmon resonance

The real time interaction and binding affinity between MtlR and HPr were measured by SPR detection using a BIAcore 3000 (BIAcore AB) as described previously (Lee *et al.,* 2007). Purified HPr was immobilized on the carboxymethylated dextran surface of a CM5 sensor chip using a NHS/EDC reaction. The standard running buffer was 20 mM HEPES pH 7.4, 100 mM NaCl. Different amount of MtlR was introduced at a flow rate of 10 μl/min. The K_D value between MtlR and HPr was determined using BIA evaluation 2.1 software.

5. Determination of the phopshorylation state of EIIA^{Glc}

The phosphorylation state of EIIA^{Glc} was determined using a protocol by Hogema *et al* (1998). 1 ml of cell culture at various growth stage were centrifuged at 16,000 × g for 2 min and discard 900 μl of culture medium. Cells were resuspended with 100 μl of remaining medium and quenched by adding 50 μl of 5 M NaOH followed by vortexing for 30 sec. 150 μl of 3 M sodium acetate (pH 5.2) and 1 ml of 100% ethanol were added. After inverting few times, samples were chilled at –80°C for at least 1 hr, thawed and centrifuged at 16,000 × g for 30 min at 4°C. The pellet was rinsed with 500 μl of cold TDW and resuspended in 20 μl of SDS sample buffer. The same amount of sample proteins was loaded onto 16% SDS-polyacrylamide gel followed by Western blotting using anti-EIIA^{Glc} serum raised in mice as described previously (Koo *et al.,* 2004). Detection and quantification of the signals of the ratio of phosphorylated/dephosphorylated EIIA^{Glc} were done using Multi Gauge 3.1 software.

6. Determination of the phosphorylation state of HPr

Phospho-histidine residues are known to be very unstable at neutral and acidic pH; thus, exposure of samples to pH ≤9.0 was minimized. The phosphorylation state of HPr was determined using a procedure by Park *et al* (2013). 0.2% sugar was added in culture medium at A_{600} 0.5. After 30 min cultivation at 37°C, 0.2 ml of cell culture was quenched by adding 20 μl of 5 M NaOH followed by vortexing for 20 sec. 60 μl of 3 M Sodium acetate (pH 5.2) and 900 μl of ethanol were added. After inverting few times, samples were centrifuged at 16,000 × g for 10 min. The pellet was resuspended in 20 μl of urea native sample buffer. Equal amount of protein was loaded onto 16% native-polyacrylamide gel followed by Western blotting using anti-HPr serum. Phosphorylation-dependent mobility shift (PDMS) of HPr on native gel were observed as described previously (Park *et al.,* 2013).

7. One-step inactivation of chromosomal genes using pKD46 plasmid

To generate the isogenic *mtlR* deletion mutant, pKD46 plasmid was used (Datsenko *et al.,* 2000). pKD46 plasmid carries the phage λ Red recombinase, which is synthesized under the control of an inducible promoter. Primers with 55-nt extensions that are homologous to regions adjacent to the target gene to be inactivated was used to generate PCR product. Electroporation was performed to made the *mtlR* disruption strain by

using the PCR product. To increase the competency of cells, competent cell of MG1655/pKD46 was prepared using a procedure by Shi *et al.* (2003). Mutant of the *mtlR* gene was isolated as antibiotic-resistant colonies because pKD46 plasmid carries antibiotic resistance gene (*bla*). After isolation of antibiotic-resistant colony, isolated colonies were cultivated in 37°C to cure pKD46 plasmid.

8. Sugar preference test

Cells were cultivated in M9 minimal media containing 0.04% glucose (or N-acetyl-glucosamine) and 0.08% mannitol. Seed cells were overnight cultivated in LB media and washed with M9 minimal media before innoculation. Cells were inoculated in 100 ml M9 minimal media and 1 ml of cells were harvested in every 2 hr. To remove cell, centrifugation was performed at 16,000 × g for 2 min. After centrifugation, 900 μl of supernatant were transferred to new 1.5 ml tube. Remaining sugars in culture medium without cell were analyzed by HPLC (High-performance liquid chromatography). To analyze glucose (or N-acetyl-glucosamine) and mannitol, sugar-PAK column was used. During the experiment, column were maintained at 75°C and TDW was used as solvent. Remaining sugars (glucose, NAG or mannitol) were detected by RI and quantified by Dionex U3000 program.

9. Quantitative Real-time PCR

Total RNA was prepared using RNeasy Mini Kit (Qiagen) from cells grown to mid

exponential phase in LB medium and genomic DNA was removed using RNase-free DNase I (Promega). After RNA clean up, 2500 μg of RNA was used to synthesize cDNA using RNA to cDNA EcoDry™ Premix (Clontech). Gene specific primers and 2X SYBR Premix Ex Taq II (Takara) were used with 100 fold diluted cDNA in qRT-PCR analysis. Amplification and detection of product were performed using CFX96 Real-Time System (Bio-Rad). *rrsH* was used as a normalization control.

10. Electrophoretic Mobility Shift Assay

The *mtl* promoter DNA fragments from –329 to –149 and from –142 to +18 relative to the transcription start site (TSS) was amplified by PCR. The purified DNA was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. After the kinase reaction, labeled DNA was purified by centrifugation using MicroSpin™ G-25 columns (GE Healthcare). Binding reactions were performed with approximately 10 fmol of labeled *mtl* promoter DNA fragment and 13 ng of cell crude or various concentrations of purified MtlR in 20 μl of the reaction buffer (20 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM DTT, 0.1 mg BSA, 5% glycerol and 0.1 ug of poly (dI-dC)), with or without 5 mM glucose. Following incubation at room temperature for 20 min, the binding mixture was subjected to electrophoresis at room temperature on a 5% polyacrylamide gel at 120 V in TB buffer (89 mM Trizma base, 89 mM boric acid). After electrophoresis, the gel was dried and exposed to imaging plate for quantification with a bio-imaging analyzer (BAS-2500; FUJIFILM).

11. Competition test

Various forward primers carrying an NdeI site, and reverse primers harboring XhoI site were used in PCR to amplify DNA sequences of the *mtl* operon promoter. This DNA fragment was digested with NdeI and XhoI, and then cloned into the corresponding sites of pET43.1a. This gave rise to diverse versions of plasmid constructs, which encompass various DNA regions of the *mtl* operon promoter. Wild type *E. coli* MG1655 was transformed with various plasmid constructs bearing the upstream region of the *mtl* operon. As a control, the same strain was transformed with the parental plasmid pET43.1a, which lacks the *mtl* regulatory region. Total RNA was prepared from these strains and the expression level of the *mtl* operon was quantified by qRT-PCR as described above.

12. Crystallization

Crystal was obtained by vapour diffusion using a sittin-drop setup in 96-well plates and a crystal screen HT (Hampton Research) at 303 K. This was performed by mixing 0.2 μl protein complex solution (60 mg ml^{-1}) with 0.1 μl well solution (0.1 M Tris-HCl, pH 8.5, 0.2 M magnesium acetate, 15% polyethylene glycol (PEG) 8000) and equilibrating against 70 μl well solution. The crystal from the crystallization trial was used to collect X-ray diffraction data on beamline 5C – SB II of the Pohang Accelerator Laboratory (PAL). Republic of Korea using a Quantum 270 CCD detector (ADSC). The

diffraction data set was processed and scaled to 3.4 A° resolution. The crystal belonged to space group I222.

13. SEC-MALS

The MtlR-HPr complex was analyzed by SEC using a superdexTM 200 increase 5/150 GL column (GE healthcare Life Sciences) on Agilent Technologies 1260 Infinity. The mobile phase of buffer A (20 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 0.05% β-mercaptoethanol, 5% glycerol) was applied at a flow rate of 0.2 mL/min. The signals of UV, light scattering and refractive index were respectively monitored by UV/RI detector (Agilent Technologies 1260 Infinity) and miniDAWN TREOS (Wyatt). The data was processed by ASTRA 6.1 (Wyatt).

Chapter III

Results

1. The preferential utilization of glucose over mannitol is independent of EIIA^{Glc}.

1.1. Inducer exclusion is not a major factor that determines the preferential utilization of glucose over mannitol.

Because the currently accepted model of CCR postulates that dephosphorylated EIIA^{Glc} impedes the transport and metabolism of less favored sugars, I assumed that glucose preference over mannitol could also be determined by inducer exclusion and induction prevention. To verify this assumption, I performed a sugar preference test in an *E. coli* mutant deleted of the *crr* gene encoding EIIA^{Glc} (Fig. 3). It is known that glucose can be transported either by the glucose PTS or by the mannose PTS (encoded by *manXYZ*) in *E. coli* and other enteric bacteria, although the mannose PTS can be less efficient than the glucose PTS in uptake and phosphorylation of glucose in *E. coli* (Postma *et al.*, 1993; Stock *et al*., 1982). While the glucose PTS-defective (*crr* and *ptsG*) mutants could still ferment glucose and mannose, mutants with defects in both PTSs were unable to grow on glucose or mannose, implying that glucose can be transported via the mannose PTS when the glucose PTS was blocked (Stock *et al*., 1982; Curtis *et al.*, 1982). In agreement with this earlier observation, the *crr* mutant could grow on glucose, although its growth rate was slightly reduced compared to an otherwise isogenic wild-type (WT) MG1655 strain (Fig. 3A and B). As previously reported (Lengeler *et al.*, 1978; Reizer *et al*., 1992), glucose was preferred over mannitol in the WT strain (Fig. 3A). Surprisingly, glucose was still used in preference to

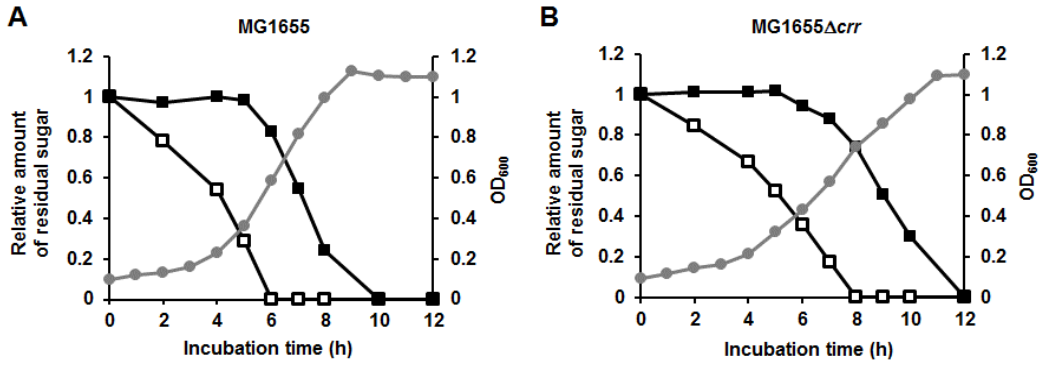

Figure 3. The preferential utilization of glucose over mannitol is independent of the *crr* mutation. Wild type and a *crr* deletion mutant of the *E. coli* K12 strain MG1655 were grown in M9 minimal medium supplemented with 0.04% glucose and 0.08% mannitol with shaking at 200 rpm. Growth rates (optical density at 600 nm, gray lines with circles) and the concentrations of sugars (open squares for glucose and closed squares for mannitol) remaining in the medium were then measured as a function of incubation time as described in the Materials and Methods section. Representative data from three independent and reproducible measurements are shown.

mannitol in the *crr* mutant (Fig. 3B). This observation therefore indicates that the preferential utilization of glucose over mannitol is independent of inducer exclusion by $EIIA^{Glc}$ and also rules out the possibility that the hierarchical utilization among PTS sugars is due to competition between various EIIs for phospho-HPr (Postma *et al.*, 1993).

1.2. Induction prevention is not a determining factor for the glucose preference.

Because the *crr* mutant is known to sustain significantly lower cAMP levels compared to the WT strain (Feucht *et al*., 1980; Nelson *et al*., 1982), it may be argued that glucose preference over mannitol in the *crr* mutant could be due to a decreased expression of the *mtl* operon which is known to belong to the CRP regulon (Figge *et al.*, 1994). Therefore, I tested the effect of induction prevention on glucose preference over mannitol. Even with the addition of exogenous cAMP to 5 mM, the glucose inhibition of mannitol utilization was still apparent in both WT and *crr* mutant strains (Fig. 4A and B), even though the addition of cAMP slightly decreased the growth rate of the two strains as previously reported (Satishchandran & Boyle, 1984). These data suggest that the induction prevention is not responsible for glucose preference over mannitol. It should be noted that previous studies have also demonstrated that the addition of exogenous cAMP did not prevent the preferred utilization of glucose over lactose and melibiose while it abolished the diauxic lag (Ann & Fraser, 1982; Okada *et al*., 1981).

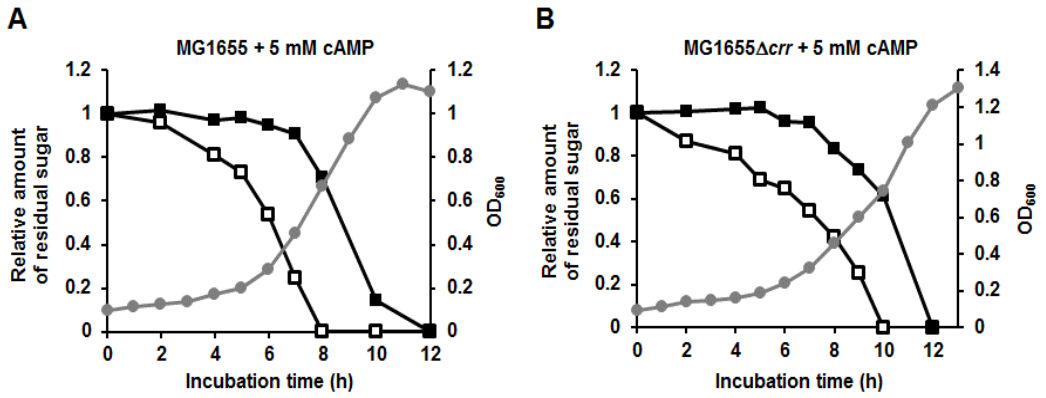

Figure 4. The preferential utilization of glucose over mannitol is independent of cAMP. Wild type and a *crr* deletion mutant of the *E. coli* K12 strain MG1655 were grown in M9 minimal medium supplemented with 0.04% glucose and 0.08% mannitol in the presence of 5 mM cAMP with shaking at 200 rpm. Growth rates (optical density at 600 nm, gray lines with circles) and the concentrations of sugars (open squares for glucose and closed squares for mannitol) remaining in the medium were then measured as a function of incubation time as described in the Materials and Methods section. Representative data from three independent and reproducible measurements are shown.

2. Glucose preference over mannitol is accomplished by repression of the *mtl* operon.

2.1. The repression effect of glucose on mannitol operon was abolished in the *mtlR* deletion mutant.

The *mtl* operon consists of *mtlADR*, where *mltA* encodes the mannitol PTS permease ($EIIABC^{Mtl}$), and *mtlD* encodes mannitol-1-phosphate 5-dehydrogenase converting mannitol-1-phosphate to fructose-6-phosphate in a reversible reaction, and *mtlR* encodes the transcriptional repressor of the *mtl* operon (Figge *et al.*, 1994). Although MtlR lacks any DNA-binding structural motifs and therefore its binding to a specific DNA operator has never been shown, the inactivation of MtlR leads to derepression of the *mtl* operon (Figge *et al.*, 1994; Tan *et al*., 2009). It is generally accepted that hierarchical utilization of carbon sources is accomplished by repression of the synthesis of the enzymes necessary for the transport and/or metabolism of less favored carbon sources in the presence of a preferred sugar (Deutscher *et al*., 2006; Görke & Stülke, 2008). For example, in enteric bacteria, the inactivation of LacI relieves the repression of the *lac* operon and eliminates glucose repression (Inada *et al*., 1996). To test whether this is also true for glucose preference over mannitol, I constructed a mutant lacking MtlR. As previously shown (Lengeler & Steinberger, 1978; Lengeler & Steinberger, 1978), the expression of *mtlA* was derepressed in the presence of mannitol alone, whereas it was repressed in the presence of glucose (Fig. 5A). Moreover, glucose significantly decreased the inductive effect of mannitol on the *mtlA* expression. Therefore, I

examined whether the inactivation of MtlR leads to derepression of the *mtl* operon and thereby influences the preference of glucose over mannitol in a similar manner as the inactivation of LacI abolishes the glucose-lactose diauxie. While glucose dramatically decreased the inductive effect of mannitol on the *mtlA* transcription in a WT strain, this glucose effect was completely abolished in the *mtlR* mutant strain (Fig. 5B). This observation led us to assume that the preferential utilization of glucose over mannitol might be related to an increase in the repressor activity of MtlR in the presence of glucose.

To verify this assumption, I first examined the effect of *mtlR* mutation on the sugar preference. As previously reported (Lengeler & Steinberger 1978; Reizer *et al.*, 1992), glucose was preferred over mannitol in the WT strain (Fig. 6A). However, the inactivation of MtlR completely abolished glucose preference over mannitol (Fig. 6B), implying that the glucose inhibition of mannitol utilization is accomplished by repression of the *mtl* operon in the presence of glucose.

2.2. Effect of the overexpression of the mannitol operon genes on the preferential utilization of glucose over mannitol.

To test the effect of the expression level of genes required for the transport and metabolism of mannitol on the hierarchical utilization of PTS sugars, sugar preference was tested with WT strains harboring a pBR322-derived expression vector for MtlA, MtlD, or both under the control of the constitutive *cat* promoter of pACYC184. As expected, the preferential utilization of glucose over mannitol was observed in a WT

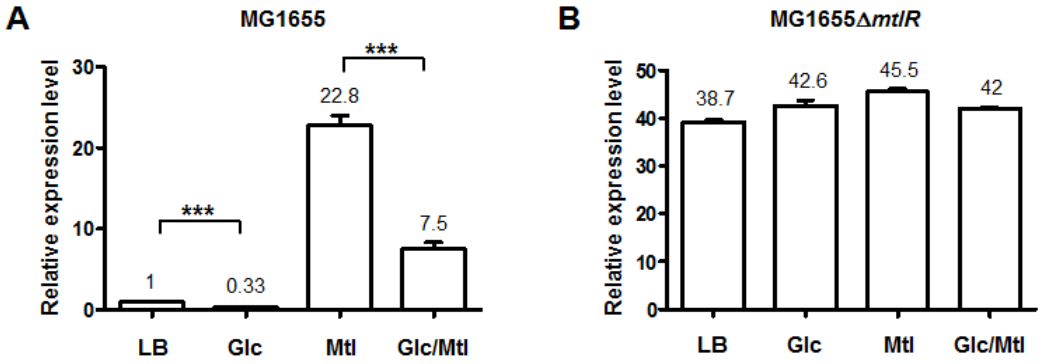

Figure 5. Inactivation of *mtlR* abolishes the glucose-dependent repression of the *mtl* operon. Total RNA was isolated from Wild type (*A*) and an *mtlR* deletion mutant (*B*) of *E. coli* MG1655 grown to early exponential phase in LB medium or LB medium containing the indicated sugars (0.2% each when added alone, or 0.1% each when added in combination), and the expression level of the *mtl* operon was then quantified by qRT-PCR as described in the Materials and Methods section. Representative data (mean ± SD) from three independent experiments (n=3 each) are shown. Statistical significance was determined by Student's *t*-test (***, $P < 0.001$).

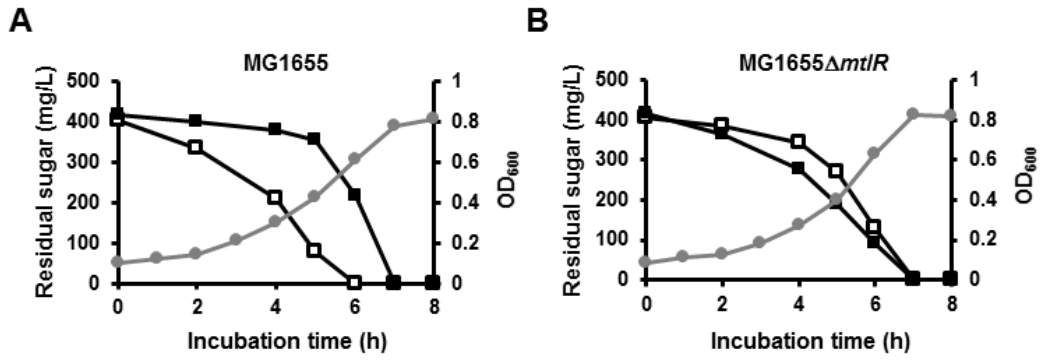

Figure 6. Inactivation of *mtlR* abolishes the glucose preference over mannitol. Wild type (A) and an *mtlR* deletion mutant (B) of *E. coli* MG1655 were grown in M9 minimal medium supplemented with 0.04% glucose and 0.04% mannitol. Growth rates (optical density at 600 nm, gray lines with circles) and the concentrations of sugars (open squares for glucose and closed squares for mannitol) remaining in the medium were then measured as a function of incubation time. Experiments were repeated at least three times with reproducible results.

strain harboring pBR322 (Fig. 7A). However, similar to the mutant lacking *mtlR* (Fig. 6B), glucose preference was completely abolished in the strain constitutively expressing MtlA alone (Fig. 7B). The constitutive expression of MtlD alone resulted in moderate growth retardation but minimally affected glucose preference (Fig. 7C). The strain expressing both MtlA and MtlD also displayed retarded growth, but the sugar preference was completely reversed in this strain (Fig. 7D). Although I am not able to explain the reason for the growth retardation by constitutive MtlD expression at present, our results support that the preference of glucose over mannitol in *E. coli* is due to the repression of the *mtl* operon, especially to a decreased expression of *mtlA*, in the presence of glucose.

3. Dephosphorylated HPr specifically interacts with MtlR.

3.1. Ligand fishing experiment shows specific interaction between MtlR and HPr.

Glucose repression of the *mtl* operon may be accomplished either by regulating MtlR itself, as shown for the regulation of the Mlc activity by the phosphorylation state-dependent interaction with the glucose PTS permease $EIICB^{Glc}$ (Lee *et al*., 2000; Nam TW *et al.*, 2001), or by regulating the transport (by MtlA) and/or metabolism (by MtlD) of mannitol, as exemplified by the regulation of sugar permeases by the phosphorylation state-dependent interaction with $EIIA^{Glc}$ (Okada *et al*., 1981; Osumi & Saier, 1982). To

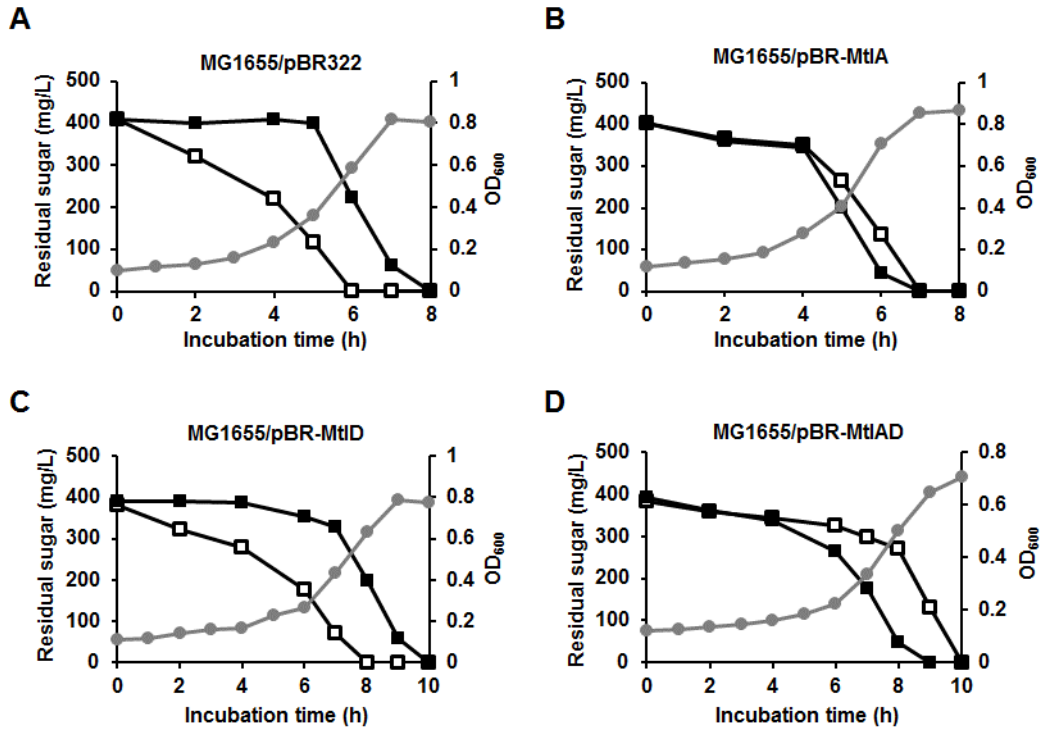

Figure 7. Effect of the overexpression of the *mtl* operon genes on the sequential utilization of glucose and mannitol. Wild-type strains harboring the pBR322 control vector (*A*) or the pBR322-derived expression vector for MtlA (*B*), MtlD (*C*) or both (*D*) were grown in M9 minimal medium supplemented with 0.04% glucose and 0.04% mannitol. Growth rates (optical density at 600 nm, gray lines with circles) and the concentrations of sugars (open squares for glucose and closed squares for mannitol) remaining in the medium were then measured as a function of incubation time. Representative data from three independent experiments are shown here.

search for a possible target mediating the glucose-dependent repression of the *mtl* operon, I constructed in-frame deletion mutants of each of the *mtl* operon genes. While the repression of the *mtl* operon by glucose was abolished in *mtlR* and *mtlA* deletion strains, it was still maintained in the *mtlD* mutant strain (Fig. 8). Therefore, I excluded the possibility of MtlD as the target mediating the glucose repression.

To further pinpoint the specific target mediating the glucose effect, protein ligand fishing experiments were performed using the N-terminally His_6-tagged MtlR and the cytoplasmic domain of MtlA (van Weeghel *et al*, 1991) as bait, as previously described (Kim *et al*., 2011; Kim *et al*., 2015; Kim *et al*., 2010; Lee *et al*., 2007; Lee *et al*., 2014). Crude lysate prepared from *E. coli* MG1655 cells was mixed with TALON metal affinity resin (Clontech Laboratories, Inc.) in the presence or absence of His-tagged bait and then subjected to pull-down assays. After brief washes, proteins bound to the resin were eluted with 150 mM imidazole. Analysis of the eluted proteins by SDS-PAGE followed by staining with Coomassie brilliant blue revealed that a protein migrating near the 6.5-kDa protein standard was observed only in the fraction containing His-MtlR as bait (Fig. 9). Interestingly, I could not detect any protein eluted exclusively in the MtlA-containing fraction (lanes 1 and 2 in Fig. 9). Peptide mass fingerprinting by MALDI-TOF MS analysis after in-gel tryptic digestion identified the protein specifically eluted with MtlR as the histidine-containing phosphocarrier protein HPr of the PTS. PTS-mediated regulatory functions are usually based on the phosphorylation

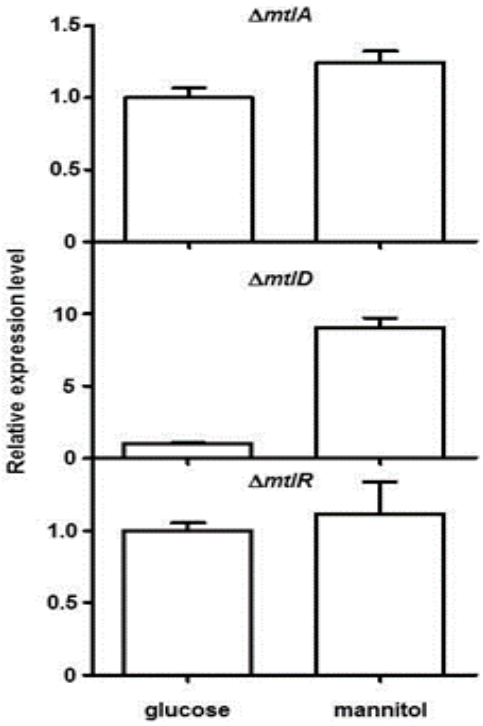

Figure 8. Effect of each gene in the *mtl* operon on the glucose-mediated repression of the operon. Deletion mutants lacking each gene in the *mtl* operon were grown in LB medium containing the indicated sugars (0.2% each), harvested at early exponential phase and the expression level of the *mtl* operon was then quantified by qRT-PCR. While the expression level of *mtlD* was measured in the *mtlA* deletion mutant, the expression level of *mtlA* was measured in *mtlD* and *mtlR* deletion mutants. Representative data (mean ± SD) from three independent experiments (n=3 each) are shown.

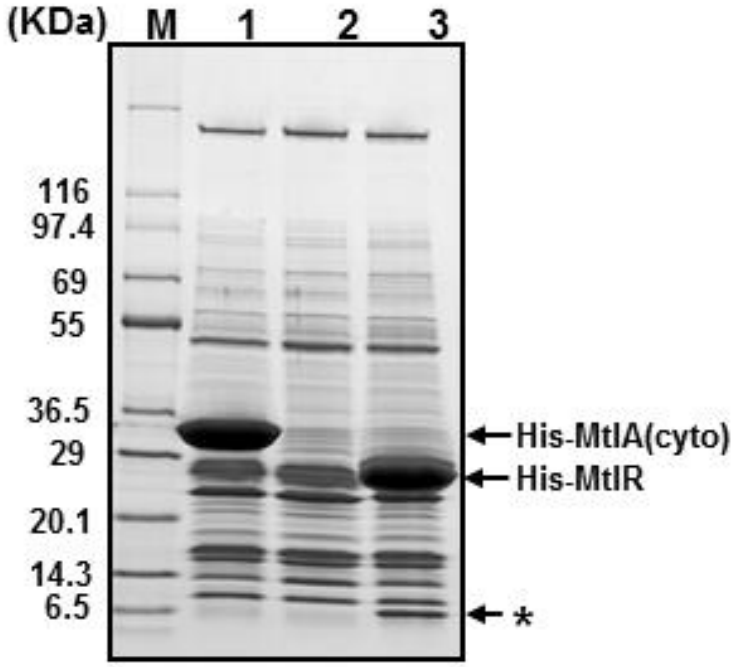

Figure 9. Specific interaction of MtlR with HPr. Protein ligand fishing experiment was performed to search for protein(s) interacting with MtlR and the cytosolic region (residues 348-637) covering the EIIA and EIIB domains of MtlA. The cell lysate prepared from *E. coli* MG1655 grown in 500 ml of LB was divided into three aliquots and mixed with buffer A (lane 2), 500 μg of the purified cytosolic region of MtlA with a His tag at the N-terminus (His-MtlA(cyto), lane 1), and 400 μg of purified His-MtlR (lane 3), respectively. The protein band bound specifically to His-MtlR is indicated by an asterisk.

state-dependent interactions with their target proteins (Deutscher *et al*., 2014; Kim *et al*., 2015; Park *et al*., 2013).

3.2. Confirmation of interaction between MtlR and HPr using talon metal affinity resin.

To confirm the specific interaction between HPr and MtlR and to test whether this interaction could be affected by the phosphorylation state of HPr, I purified untagged MtlR and His-HPr. Because it is well-established that HPr can be phosphorylated *in vitro* by incubation with EI and PEP (Parl *et al*., 2013), MtlR was mixed with EI and increasing amounts of His-HPr in the absence or presence of PEP and subjected to TALON metal affinity chromatography. As shown in Fig. 10, a concentration-dependent interaction of MtlR was observed in the absence, but not presence, of PEP, indicating that only the dephosphorylated form of HPr can interact with MtlR.

3.3. Measurement of the dissociation constant between MtlR and HPr using Surface Plasmon Resonance.

To determine the dissociation constant (K_D) for the binding of MtlR to HPr, HPr was immobilized on a CM5 sensor chip and various concentrations of MtlR were applied to the HPr surface to conduct surface plasmon resonance (SPR) spectroscopy with a BIAcore 3000 system (GE Healthcare Life Sciences) (28). The SPR signal increased as a function of MtlR concentration (Fig. 11). The K_D for the MtlR-HPr interaction was

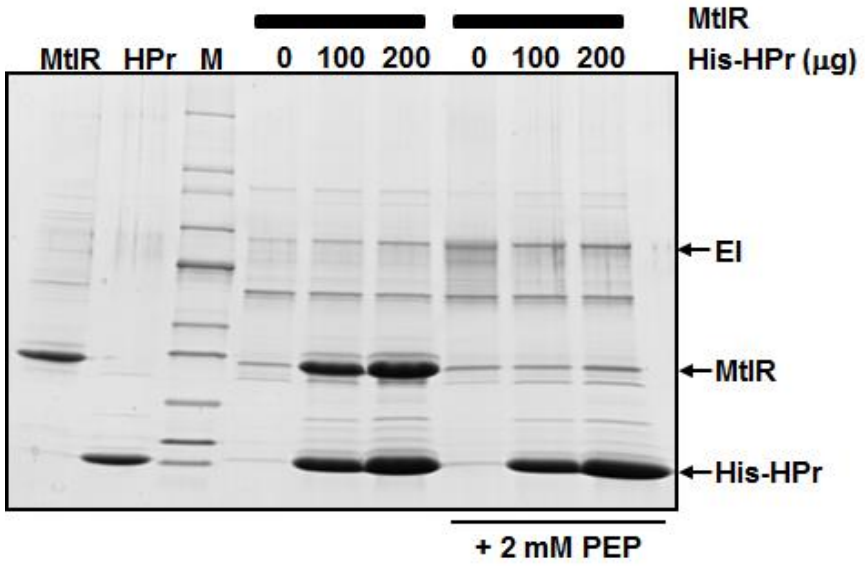

Figure 10. Dephosphorylated HPr specifically interacts with MtlR. Untagged MtlR (200 μg) was mixed with 5 μg of EI, 50 μl of TALON metal affinity resin, and various amounts of His-HPr (0, 100, 200 μg) in the absence or presence of 2 mM PEP as indicated. These mixtures were then subjected to pull-down assays to examine the specificity of the interaction between MtlR and HPr and its dependence on the phosphorylation state of HPr. After agitation for 10 min at 4°C, the protein-bound resin was washed with buffer A containing 10 mM imidazole and eluted with 50 μl of 2X SDS loading buffer. The eluted protein samples (20 μl each), along with MtlR and His-HPr proteins, were analyzed by 4-20% SDS-PAGE.

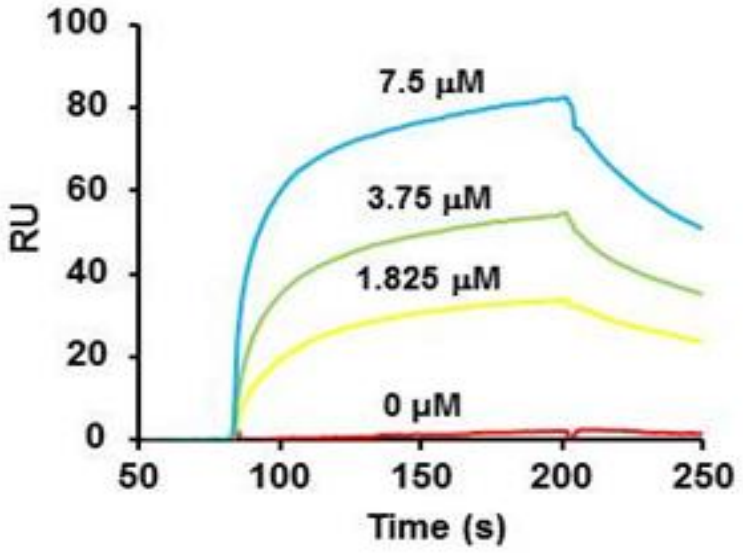

Figure 11. Measurement of the dissociation constant (K_D) between HPr and MtlR. Real-time interaction of MtlR and HPr was monitored by surface plasmon resonance (SPR) detection using a BIAcore 3000 (GE Healthcare Life Sciences) as previously described with some modifications (Kim *et al*., 2015). Purified HPr was immobilized on the carboxymethylated dextran surface of a CM5 sensor chip using a NHS/EDC reaction. The standard running buffer was 20 mM HEPES (pH 7.4) and 100 mM NaCl. All reagents were introduced at a flow rate of 10 μl/min. The indicated amounts of MtlR were allowed to flow over immobilized HPr. The sensor surface was regenerated between assays by flushing with the standard running buffer at a flow rate of 100 μl/min for 10 min to remove bound analytes. The K_D value between MtlR and HPr was determined using BIAevaluation 2.1 software (GE Healthcare Life Sciences).

determined using BIAevaluation software to be approximately $6.22x10^{-8}$ M, assuming a 1:1 interaction.

3.4. Determination of the binding stoichiometry between MtlR and HPr by using a gel filtration column.

To confirm the tight interaction between HPr and MtlR and to determine the binding stoichiometry, I compared the elution profile from a gel filtration column (Superose 12 10/300 GL; GE Healthcare Life Sciences) of the complex with the profiles of individual proteins (Fig. 12). MtlR alone was eluted as a symmetric peak at approximately 11.6 ml, corresponding to the tetrameric form (monomer = approximately 22.0 kDa), whereas HPr (approximately 9.1 kDa) was eluted at approximately 14.5 ml. When the mixture of MtlR and HPr was subjected to gel filtration chromatography, the peak at approximately 11.6 ml was shifted to approximately 11.3 ml, with a concomitant slight decrease in the peak at approximately 14.5 ml (Fig. 12A). When the gel filtration fractions were analyzed by SDS-PAGE, those eluted at approximately 11.3 ml were resolved into two bands migrating at the positions expected for HPr and MtlR (Fig. 12B). Based on the band intensities of the two proteins in these fractions, it appears that HPr interacts with MtlR in a 1:1 ratio and forms a heterooctamer. The low K_D value obtained using the BIAcore 3000 system explains that the MltR-HPr complex was tight enough to survive during gel filtration chromatography. It has been already reported that MtlR forms heterodimer which is far different from globular structure (Tan *et al*., 2009), MtlR-HPr

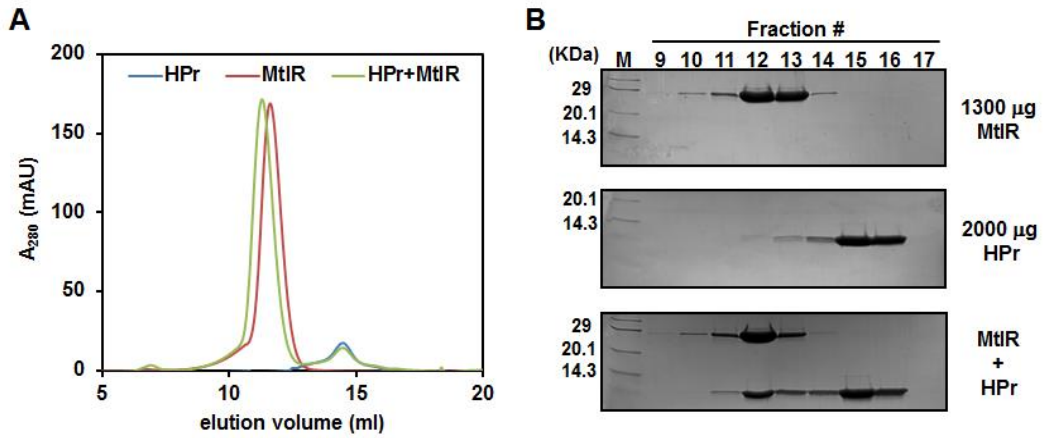

Figure 12. Gel filtration chromatography of MtlR, HPr and the MtlR-HPr complex. Gel filtration chromatography was performed on a Superose 12 10/300 GL column (GE Healthcare Life Sciences) equilibrated with running buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 0.05% β-mercaptoethanol and 5% glycerol at a flow rate of 0.5 µl/min using AKTA FPLC system. (*A*) Each sample containing 1300 µg of MtlR (red line), 2000 µg of HPr (blue line), or both proteins (green line) in 200 ml of the running buffer was injected through the column and the three chromatograms recorded at 280 nm were superimposed to compare the elution profiles. (*B*) Fractions (1 ml) were collected, and 25 µl of each fraction was analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue R-250. Lane M indicates the EZway^{TM} Protein Blue MW Marker (KOMABIOTECH), and the molecular masses (in kDa) of some standards are presented on the left. Numbers indicate fractions from the gel filtration column.

complex could be eluted earlier in gel filtration chromatography. To verify the stoichiometry of MtlR-HPr complex in detail, MALS experiment was performed. One clean peak was detected and the molecular weight of complex was calculated based on the data collected by UV and refractive index (RI) detector. The calculated molecular mass of the MtlR-HPr complex was 63.66 kDa. Based on MALS result, in solution, at high protein concentration, the MtlR-HPr complex is likely to exist as a heterotetramer. Taken together, these data show that only the dephosphorylated form of HPr specifically interacts with MtlR in a 1:1 ratio to form a heterotetramer.

4. Dephosphorylated HPr inhibits derepression of the *mtl* operon by mannitol.

4.1. Determination of the *in vivo* phosphorylation state of HPr in the presence of various carbon sources.

I have previously shown that HPr was predominantly in the phosphorylated state in LB medium and the LB medium supplemented with a non-PTS carbon source, glycerol, whereas it was almost completely dephosphorylated in the presence of glucose (Park *et al*., 2013). Because HPr is a general PTS component commonly used for many PTS sugars, I first examined the phosphorylation state of HPr in the presence of various PTS sugars (Fig. 13). It should be noted that because the histidine residue phosphorylated at the N1 position in HPr is extremely unstable at pH < 9.0 (Park *et al*., 2013; Hultquist,

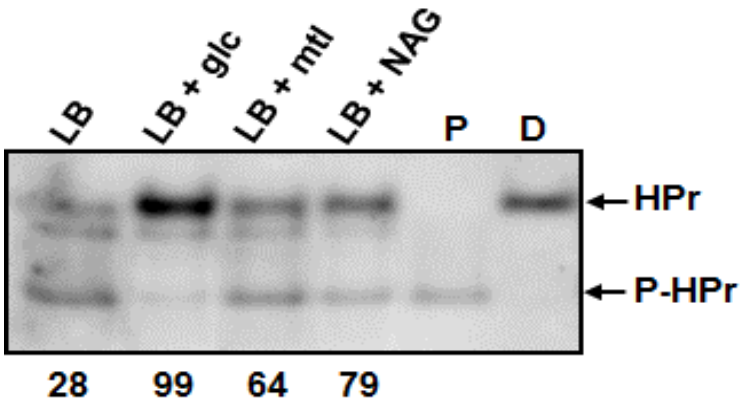

Figure 13. Determination of the *in vivo* phosphorylation state of HPr by western blotting. The wild-type K12 strain MG1655 harboring pACYC-HPr was grown in LB medium or LB medium containing the indicated sugars (0.2% each). Cells from an exponentially growing culture were harvested and the phosphorylation state of HPr was determined as described in the Materials and Methods section. Purified HPr (2 ng) was used as a positive control. Band intensities of the dephosphorylated HPr were analyzed using Multi Gauge version 3.0 software, and the percentage of dephospho-HPr over total HPr was given below the gel.

1968), exposure of samples to pH < 9.0 was minimized after the phosphorylation state of HPr was fixed and cells were lysed in culture medium without any cell harvesting step by adding NaOH (Park *et al*., 2013). Even though HPr is shared among several PTS sugars, its phosphorylation state significantly varied depending on the type of the PTS carbohydrate. While HPr existed mostly in the dephosphorylated form in the presence of glucose, approximately 21 and 36% of HPr proteins were phosphorylated in the presence of N-acetyl-glucosamine and mannitol, respectively. Because only dephosphorylated HPr could interact with MtlR and the level of dephosphorylated HPr was significantly higher in the presence of glucose compared to in the presence of mannitol, I assumed that the glucose repression of the *mtl* operon may be accomplished via the augmentation of the MtlR activity by dephosphorylated HPr.

4.2. The role of dephosphorylated HPr in the regulation of mannitol utilization.

To verify this assumption, I constructed pACYC-H15A, a pACYC184-derived expression vector for the unphosphorylatable form of HPr (HPr(H15A)) driven by the *E. coli ptsH* promoter, and compared the growth rate and the *mtl* operon expression level between the WT MG1655 strains harboring pACYC184 or pACYC-H15A (Fig. 14). While the strain harboring pACYC-H15A showed a similar growth rate with the strain harboring the control vector in medium containing glucose as the sole carbon source (compare curves marked with triangles in Fig. 14A), the expression of

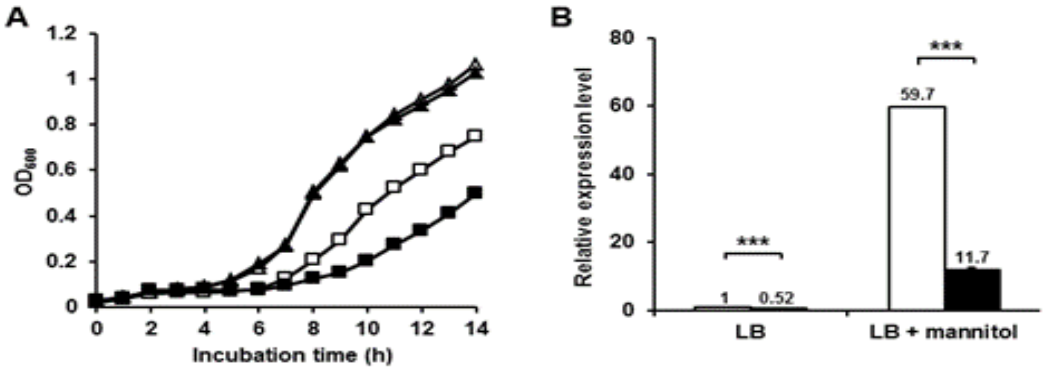

Figure 14. Dephosphorylated HPr inhibits derepression of the *mtl* operon by mannitol. (*A*) Growth of the MG1655 strain harboring the control vector pACYC184 (open symbols) or pACYC-H15A expressing an unphosphorylated form of HPr (closed symbols) was monitored in M9 minimal medium supplemented with 0.2% glucose (triangles) or mannitol (squares) as the sole carbon source at 37°C. (*B*) The wild-type *E. coli* MG1655 strain harboring pACYC184 (open bar) or pACYC-H15A (closed bar) was cultivated in LB medium or LB medium containing 0.2% mannitol, harvested at early exponential phase, and the expression level of *mtlA* was then quantified by qRT-PCR. Representative data (mean ± SD) from three independent experiments (n=3 each) are shown, and statistical significance was determined by Student's *t*-test (***, $P < 0.001$). Transcript levels are expressed relative to the strain harboring pACYC grown in LB medium.

HPr(H15A) significantly retarded the growth rate of the MG1655 strain in medium supplemented with mannitol as the sole carbon source (compare curves marked with squares in Fig. 14A). As shown in Fig. 14B, when the WT strain was transformed with pACYC-H15A, the expression of *mtlA* was significantly decreased even in the absence of glucose. Furthermore, the episomal expression of HPr(H15A) significantly decreased the inductive effect of mannitol on the *mtlA* expression. Therefore, these data suggest that glucose preference over mannitol is accomplished, at least in part, by augmentation of the transcriptional repressor activity of MtlR by dephosphorylated HPr in the presence of glucose.

4.3. Dephosphorylated HPr determines glucose and N-acetyl-glucosamine as preferential carbon sources over mannitol.

When glucose is transported into the cell, HPr becomes dephosphorylated (Fig. 13). MtlR only interacts with dephosphorylated HPr, indicating that the interaction between HPr and MtlR increases in the presence of glucose. Based on our experiments, HPr-MtlR complex, as a result of increased ratio of dephosphorylated HPr, regulates the expression of mannitol operon. To unveil the physiological effect of dephosphorylated HPr, sugar preference test between N-acetyl-glucosamine (NAG) and mannitol was performed (Fig. 15). According to our model, NAG would be preferentially metabolized

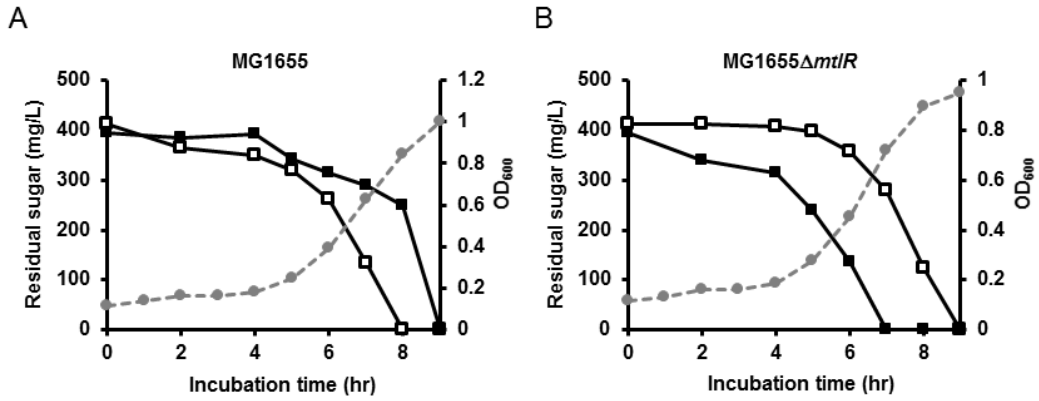

Figure 15. Increased HPr during the utilization of N-acetyl glucosamine (NAG) determines NAG as preferential carbon source over mannitol. Wild type (A) and an *mtlR* deletion mutant (B) of *E. coli* MG1655 were grown in M9 minimal medium supplemented with 0.04% NAG and 0.04% mannitol. Growth rates (optical density at 600 nm, gray lines with circles) and the concentrations of sugars (open squares for NAG and closed squares for mannitol) remaining in the medium were then measured as a function of incubation time. Experiments were repeated at least three times with reproducible results.

over mannitol as the ratio of *in vivo* HPr/P-HPr in cells grown in the presence of NAG is higher than that of cells grown in minimal media supplemented with mannitol (Fig. 13). 0.04% NAG and 0.08% mannitol were supplied in minimal media and MG1655 was inoculated. Cells were harvested every 2 hours and the amount of remaining sugars in medium was determined by HPLC. NAG was rapidly used over mannitol as in the case of glucose (Fig. 6A). Dephosphorylated HPr resulting from NAG transport forms a complex with MtlR and represses the expression of mannitol operon. As a result, utilization of NAG inhibits the transport and subsequent catabolism of mannitol same as in the presence of glucose. Repression of mannitol metabolism by NAG transport also disappeared in *mtlR* deletion mutant. In summary, sequential utilization of glucose or NAG then mannitol is due to the repression of mannitol operon expression by HPr-MtlR complex, formed when HPr is dephosphorylated.

5. The interaction between HPr and MtlR determines glucose preference over mannitol.

5.1. Screening for the interaction between MtlR and mutant HPrs.

I anticipated that if the interaction between dephosphorylated HPr and MtlR is sufficient to confer the preference of glucose over mannitol, a mutant HPr, which still retains the phosphotransferase activity to transport both glucose and mannitol but is no longer able to interact with MtlR, may abolish the preference between glucose and

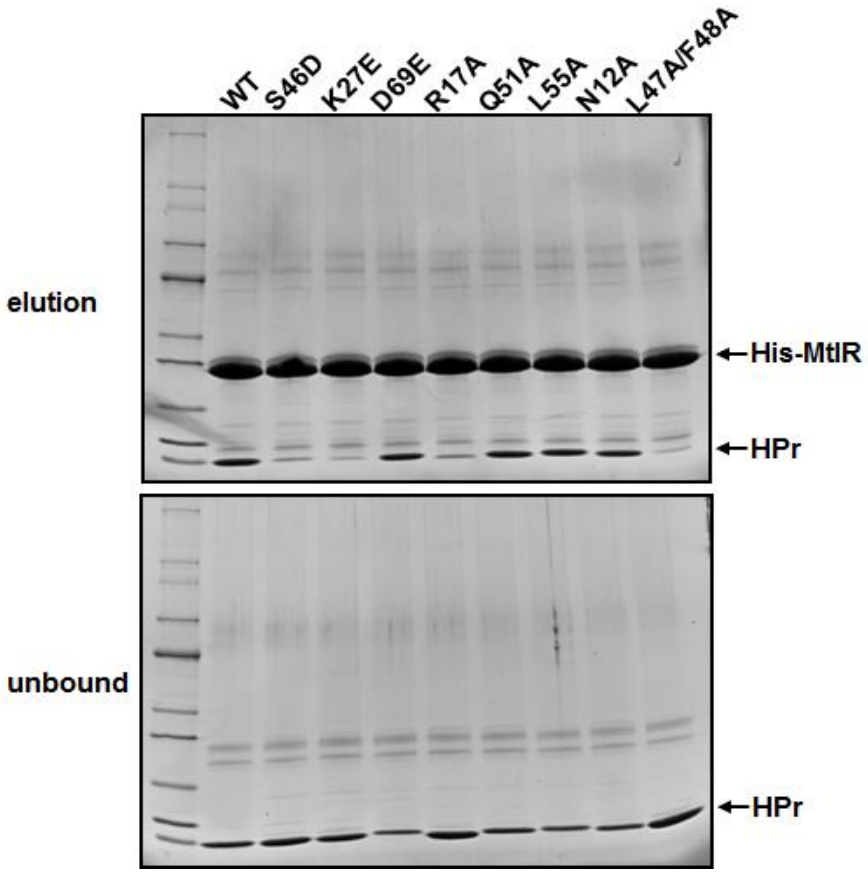

Figure 16. Examination of mutant HPrs for their interaction with MtlR. His-MtlR (100 μg) was mixed with mutant HPrs (50 μg each) and then subjected to TALON metal affinity chromatography. After a brief wash with buffer A containing 10 mM imidazole, samples were eluted with 2X SDS loading buffer. Along with and unbound samples (lower gel), eluted samples (upper gel) were analyzed by 4-20% SDS-PAGE followed by staining with Coomassie brilliant blue R-250.

mannitol. Therefore, I purified several mutant HPrs from our collection and examined their interaction with MtlR by TALON metal affinity chromatography using His-tagged MtlR as bait. As shown in Fig. 16, the interaction with MtlR was almost completely abolished or significantly decreased for several mutant forms of HPr (R17A, K27E, S46D, and L47A/F48A), while other mutants still retained the interaction with MtlR to a comparable level as the WT HPr. The Arg17 residue near the His15 phosphorylation site is highly conserved, and a previous study has shown that all mutations at Arg17 resulted in impairment of the phosphocarrier function of HPr with EI (Anderson *et al*., 1993). The Ser46 residue is also widely conserved in Gram-positive and Gram-negative bacteria and mutation of Ser46 to Asp was previously shown to decrease the phosphoacceptor activity of HPr from EI by approximately 2000-fold (Napper *et al.*, 1996). Therefore, I examined whether the phosphotransferase activity is impaired in K27E and L47A/F48A mutants of HPr.

5.2. Confirmation of phosphotransferase activity of mutant HPrs.

Therefore, I examined whether the phosphotransferase HPr by determining the growth rate of a *ptsH* deletion mutant harboring a pACYC184-derived expression vector pACYC-HPr, pACYC-HPr(K27E) or pACYC-HPr(L47A/F48A) in M9 medium containing 0.2% glucose or mannitol (Fig. 17). As previously reported (Postma *et al*., 1993), the *ptsH* mutant did not grow in M9 medium supplemented with glucose or mannitol. The expression of the L47A/F48A mutant HPr in trans could rescue the growth defect of the *ptsH* deletion strain in M9 medium supplemented with glucose, but

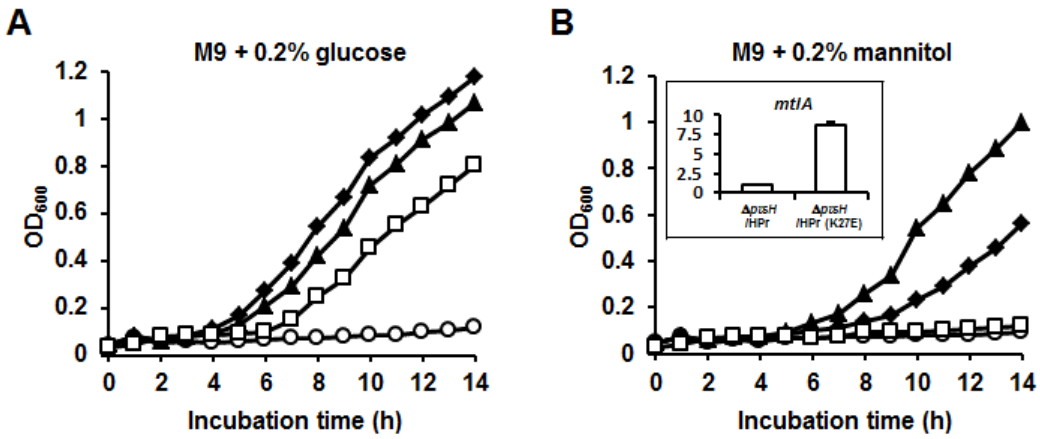

Figure 17. Confirmation of the phosphotransferase activity in mutant HPr complementation strains. Growth of a *ptsH* deletion mutant harboring a pACYC184 (open circles) or a pACYC184-derived expression vector for wild-type HPr (pACYC-HPr, closed diamonds), pACYC-HPr(K27E) (closed triangles) or pACYC-HPr(L47A/F48A) (open squares) in M9 medium containing 0.2% glucose (*A*) or 0.2% mannitol (*B*). Growth of the *ptsH* deletion mutant (open circles) is shown as a negative control. The inset in ***B*** shows the expression level of *mtlA* measured by qRT-PCR in the *ptsH* deletion mutant harboring pACYC-HPr(K27E) relative to that in the strain harboring pACYC-HPr.

not in mannitol-containing medium. The *ptsH* mutant strain harboring pACYC-HPr(K27E) showed a similar growth rate with the mutant strain harboring pACYC-HPr in glucose-containing medium. Surprisingly, however, the *ptsH* mutant strain harboring pACYC-HPr showed a significantly slower growth rate than the strain harboring pACYC-HPr(K27E) in M9 minimal medium containing mannitol even though expression levels of the two HPr proteins were quite similar. It is shown that an increase in dephosphorylated HPr represses the expression of the *mtl* operon (Fig. 14B) and approximately 64% of HPr exists in the dephosphorylated state in the presence of mannitol (Fig. 13). Therefore, I assumed that the slower growth rate of the strain expressing WT HPr from the multi-copy plasmid pACYC184 compared to the HPr(K27E)-overproducing strain in the mannitol-containing medium might be due to the augmentation of the MtlR repressor activity by WT HPr dephosphorylated during the transport of mannitol but not by HPr(K27E), which cannot interact with MtlR. Indeed, a significantly greater repression of the *mtlA* expression was observed in the WT HPr-overproducing strain than the HPr(K27E)-overproducing strain even in the presence of mannitol (inset in Fig. 17B).

5.3. Effect of the interaction between MtlR and HPr on the sequential utilization of glucose and mannitol.

Then, to determine the contribution of the tight interaction between dephosphorylated HPr and MtlR to the preference between glucose and mannitol, the sugar preference experiments were performed with HPr- and HPr(K27E)-overproducing strains. Interestingly, while glucose was still preferred over mannitol in the strain harboring pACYC-HPr (Fig. 18A), glucose preference was almost completely abolished in the strain harboring pACYC-HPr(K27E) (Fig. 18B), and this strain consumed glucose and mannitol simultaneously as did the *mtlR* deletion mutant (Fig. 6B). These data indicate that the interaction between dephosphorylated HPr and MtlR is sufficient to confer the preference of glucose over mannitol. Taken together, the data in this study show that a higher level of the dephosphorylated form of the general PTS component HPr accumulates in the presence of a more favorable sugar, and dephosphorylated HPr determines the preference between the PTS sugars glucose and mannitol by inhibiting the induction of genes required for transport and metabolism of the less preferred sugar mannitol via a direct interaction with the mannitol operon repressor MtlR.

6. A novel type of transcriptional repression mediated by MtlR.

6.1. Properties of an unidentified factor X which mediates the interaction between MtlR and the promoter of the *mtl* operon.

As would be anticipated from the absence of DNA binding motif in MtlR (Tan *et al*., 2009), it is reasonable to speculate that another player, hereafter referred to factor X,

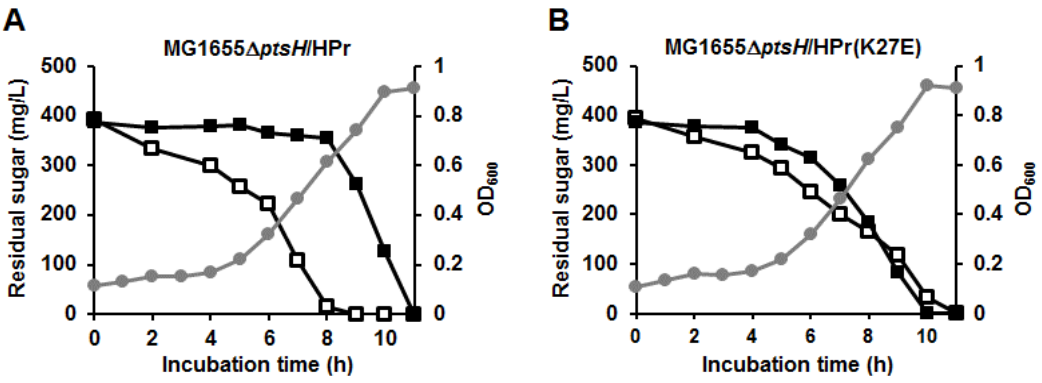

Figure 18. The interaction between HPr and MtlR is sufficient to confer glucose preference over mannitol. The *ptsH* deletion strain harboring pACYC-HPr (*C*) or pACYC-HPr(K27E) (*D*) was grown in M9 minimal medium supplemented with 0.04% glucose and 0.04% mannitol. Growth rates (optical density at 600 nm, gray lines with circles) and the concentrations of sugars (open squares for glucose and closed squares for mannitol) remaining in the medium were measured as a function of incubation time. Representative data from three independent and reproducible measurements are shown.

with the ability to bind DNA is involved in the transcriptional regulation of the *mtl* operon by MtlR. To demonstrate the existence of the factor X, I performed electrophoretic mobility shift assay (EMSA) using cell lysate. Cell lysate prepared from three different strains, mutants devoid of CRP alone or both of CRP and MtlR, and MtlR complemented in *crp* and *mtlR* double deletion strain, were incubated with *mtl* promoter region and the reaction mixtures were loaded onto the 5% polyacrylamide gel. Electrophoretic mobility shifts were observed in all three lanes but supershift was observed only when MtlR was present in the cell lysate (Fig. 19A, lane 1 and 3). This result suggests that MtlR binds to the promoter region of the *mtl* operon. Furthermore, EMSA experiment with purified MtlR was carried out to confirm the binding of MtlR to the promoter region of *mtl*. As shown in Fig. 19B, increasing amount of purified HisMtlR in the presence of cell lysate prepared from double deletion strain of *crp* and *mtlR* showed increased mobility shift. According to our EMSA data, MtlR binds to the promoter of *mtl* operon by interaction with a so-far unknown DNA binding protein.

6.2. MtlR represses the expression of the *mtl* operon through its interaction with the promoter of the *mtl* operon.

It was previously known that MtlR lacks a DNA-binding domain (Figge *et al*., 1994; Tan *et al*., 2009). Eventhough MtlR cannot directly to DNA, MtlR shows a repression effect on the expression of the *mtl* operon (Figge *et al*., 1994). To verify whether MtlR is a novel regulation machinery, a competition test was performed (Fig. 20). Based on the previously published experiment (Figge *et al*., 1994), various lengths of DNA

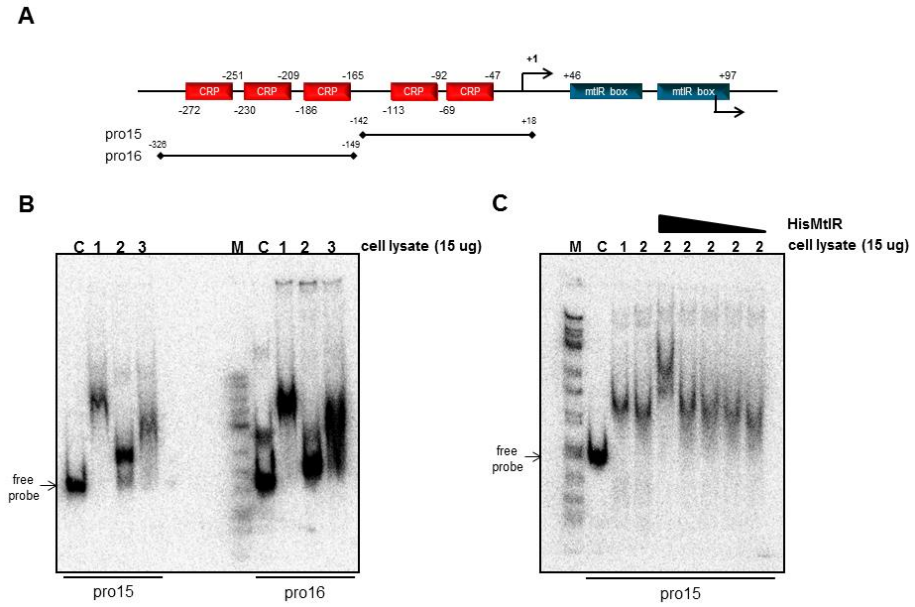

Figure 19. Electrophoretic mobility shift assay for interaction between the *mtl* promoter DNA and MtlR. Electrophoretic mobility shift assay was performed using cell lysate (B) or purified protein (C) as described in the Materials and Methods section. (A) Schematic depiction of the *mtl* operon and DNA constructs used in EMSA. (B) Interaction between MtlR and DNA was determined by using cell lysate prepared from MG1655Δ*crp* strain (lane 1), MG1655Δ*crp*Δ*mtlR* strain (lane 2) and MG1655Δ*crp*Δ*mtlR*/pBR-*mtlR* strain (lane 3). Two types of DNA fragments (pro15 and pro16) were used as probes. (C) The interactions between various concentrations of purified HisMtlR (50 uM, 10 uM, 2 uM, 400 nM, 80 nM) and DNA fragment (pro15) were observed in the presence of cell lysate. Cell lysate prepared from MG1655Δ*crp* strain (1), MG1655Δ*crp*Δ*mtlR* strain (2) were used.

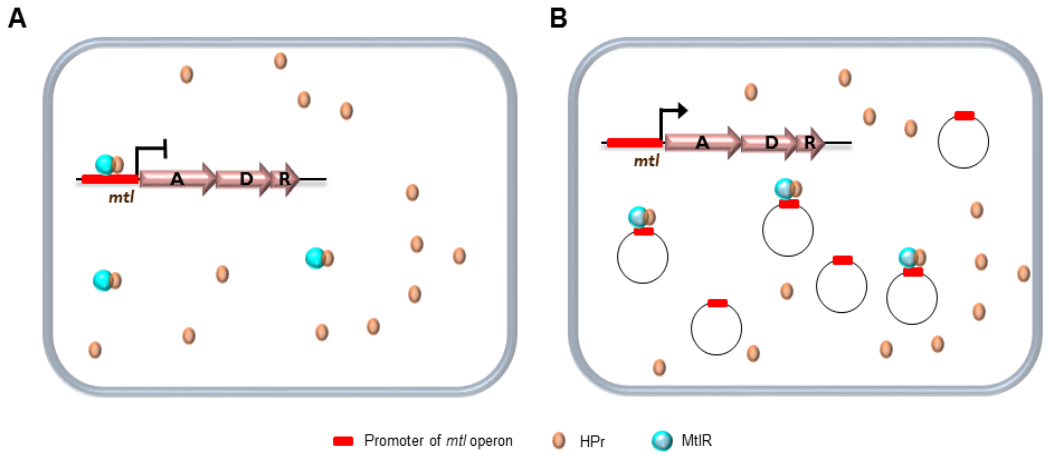

Figure 20. A schematic view of the competition assay. In wild type *E. coli* (A), MtlR-HPr complex binds to the promoter region and represses the expression of the *mtl* operon. When plasmids bearing the promoter region of the *mtl* operon is transformed into MG1655 (B), MtlR-HPr complexes are sequestered by promoter DNA in plasmid. Consequently, the expression level of the *mtl* operon increases.

constructs containing the promoter region of the *mtl* operon and upstream region of translational start site of MtlA were used to identify the binding site of MtlR. Based on the competition test performed by Figge *et al*., (1994), MtlR box was considered as a binding sequence of MtlR. However, MtlR box showed no effect in our competition test, whereas the promoter region of the *mtl* operon, composed of five CRP binding sites was identified as a binding site of MtlR (Fig. 21). Based on our data, MG1655 strain harboring DNA construct containing not only all the five CRP binding sites but partial region of promoter with more than two continuous CRP binding sites showed inducible effect of *mtlA* (Fig. 21C). Because MtlR cannot directly bind to the promoter region of the *mtl* operon, interaction between MtlR and the promoter region might be mediated by an unknown DNA binding protein (factor X).

6.3. MtlR interacts with a specific motif which only exists in the promoter region of the *mtl* operon.

Based on the result of Fig. 21, I anticipated that the binding sequence of MtlR should be located nearby the CRP binding sites. Although more than 300 CRP binding sites were identified in *E. coli* (Zheng *et al*., 2004), the repression effect of MtlR seems to appear only in the *mtl* operon. Therefore, I hypothesized that a consensus sequence for MtlR binding is located only in the promoter of the *mtl* operon. I attempted to verify this

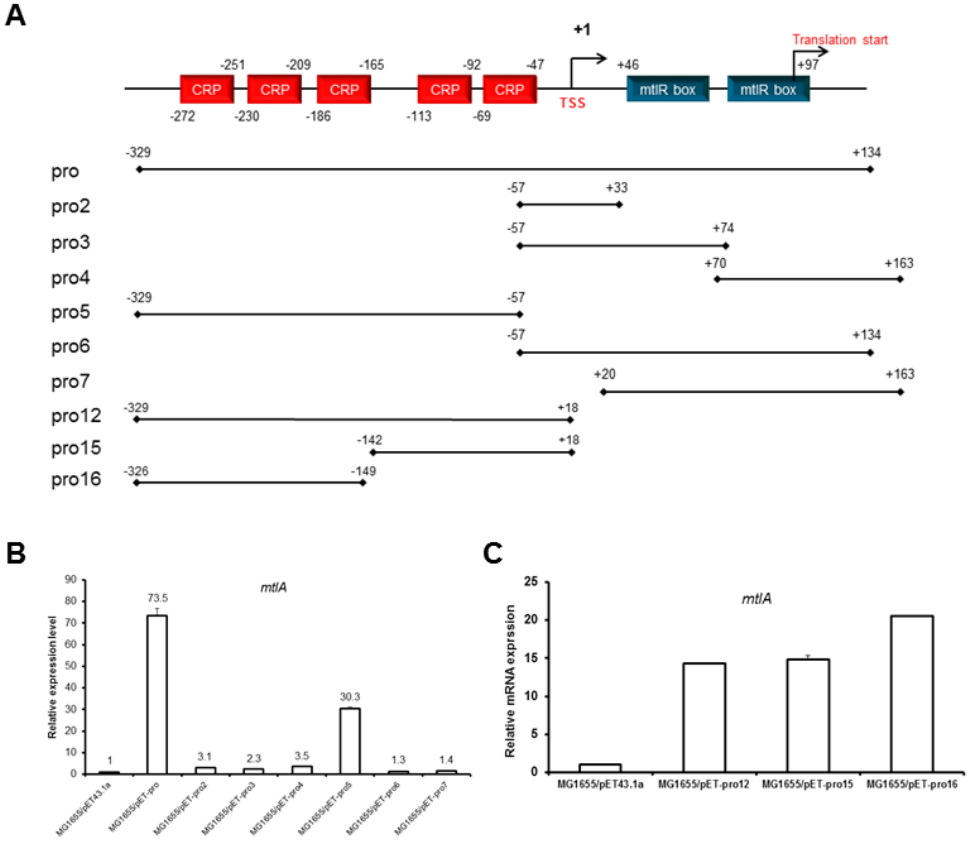

Figure 21. Expression level of the *mtl* operon in the wild type *E. coli* MG1655 harboring various plasmids. (A) Schematic depiction of the *mtl* operon and DNA constructs used in the competition test. (B-C) The expression level of *mtlA* was measured by qRT-PCR in wild type strain MG1655 harboring various DNA constructs relative to that in the strain harboring control vector (pET43.1a) was shown. Repression effect of MtlR on the expression of the *mtl* operon was decreased in the presence of promoter DNA.

hypothesis by introducing the promoter region of *malK* in wild type *E. coli* (Fig. 22). Promoter of *malK* is structurally similar to the promoter of the *mtl* operon, and also contains four CRP binding sites (Fig. 22A). As expected, transformation of MG1655 with plasmid pET-malK had no effect on the inducible expression of *mtlA* whereas pET-pro12 showed inducible effect on the expression of *mtlA* (Fig. 22B). To verify whether the regulatory effect of MtlR on the expression of the *mtl* operon is specific, the expression levels of CRP regulons were quantified by qRT-PCR in the *mtlR* deletion mutant but no meaningful change was detected. Based on these results, I concluded that the binding sequence of MtlR may exists only on the promoter of the *mtl* operon.

6.4. Properties of factor X

Although I was not able to identify the factor X, I tested several features of protein X to allow us identify it in the near future. As shown in Fig. 23, the overexpression of MtlR in the presence of pET-pro12 showed repressed expression of the *mtl* operon whereas introduction of the control plasmid (pACYC184) had inducible effect on the expression of *mtlA*. This data suggest that the expression level of the factor X may be higher than that of MtlR.

In addition to the expression level of the factor X, the binding property of the factor X can be deduced from our data. Intracellular level of the factor X is expected to be similar in strains harboring pACYC184 or pACYC-MtlR. The only difference between the two strains is the expression level of MtlR. Although it is reasonable to speculate

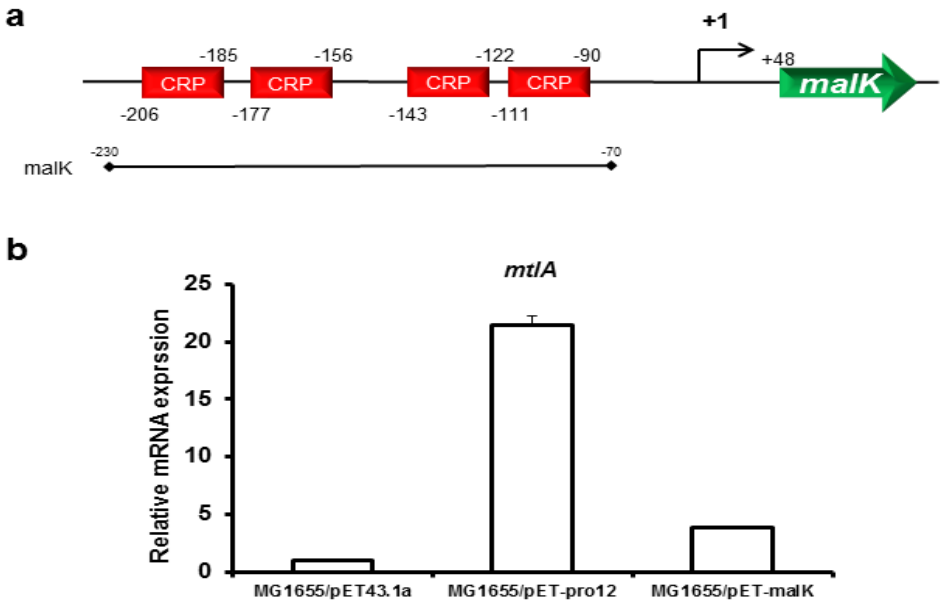

Figure 22. Effect of the *malK* promoter DNA on the expression level of the *mtl* operon. (A) Schematic depiction of the promoter region of malK and DNA construct. (B) The expression level of *mtlA* was measured by qRT-PCR in wild type strain MG1655 harboring pET-pro12 and pET-malK. The expression level of *mtlA* in MG1655 harboring control plasmid (pET43.1a) was used as a negative control.

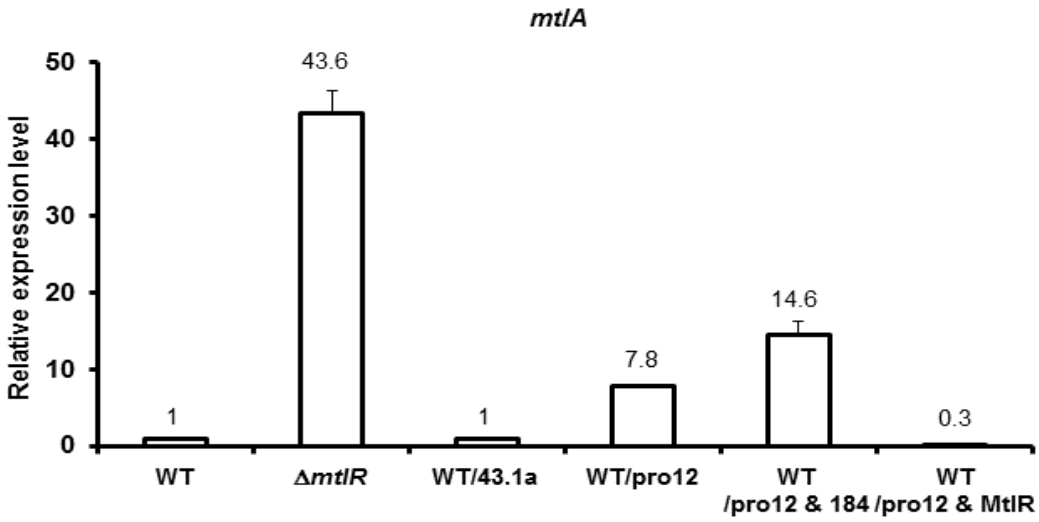

Figure 23. Effect of overexpressing MtlR in cells harboring pET-pro12 plasmid.

Indicated strains were grown in LB medium and harvested at early exponential phase. The expression level of the *mtl* operon was then measured by qRT-PCR. Representative data (mean ± SD) from three independent experiments (n=3 each) are shown.

that enough amount of the factor X was present in the strain harboring both pET-pro12 and pACYC184, the expression level of *mtlA* increased compared to wild type strain. According to this result, I guess that factor X cannot repress the expression of *mtlA* without MtlR. Although unlike MtlR, the factor X is likely to possess a DNA-binding motif, our result supports that the factor X cannot interact with the consensus sequence in the *mtl* promoter in the absence of MtlR.

7. Crystal structure of the MtlR-HPr complex.

To confirm the tight interaction of HPr with MtlR and to determine the binding stoichiometry, I solved the crystal structure of MtlR-HPr complex (Fig. 24). The crystal structure of complex was determined by the molecular-replacement method using the structures of HPr and MtlR as search model. Consistent with the results in Fig. 12, the crystal structure revealed a heterooctamer in a 1:1 ratio complex of HPr and MtlR. As expected from the binding test in Fig. 15, four residues in HPr (Arg17, Lys27, Ser46 and Lys47/Phe48) takes important part in the interaction with MtlR (Fig. 25A). The interactions are focused on a small area and include both polar and hydrophobic interactions. It has been shown that the binding between HPr and MtlR is abolished when His15 of HPr is phosphorylated (Fig. 10). His15 in HPr is positioned in the binding interface of MtlR and HPr. When His15 of HPr is phosphorylated, the bulkier residue is likely to cause severe steric hindrance at the binding interface (Fig. 25B). Comparative analysis with another crystal structure of HPr complex (HPr–Rsd) (Park *et*

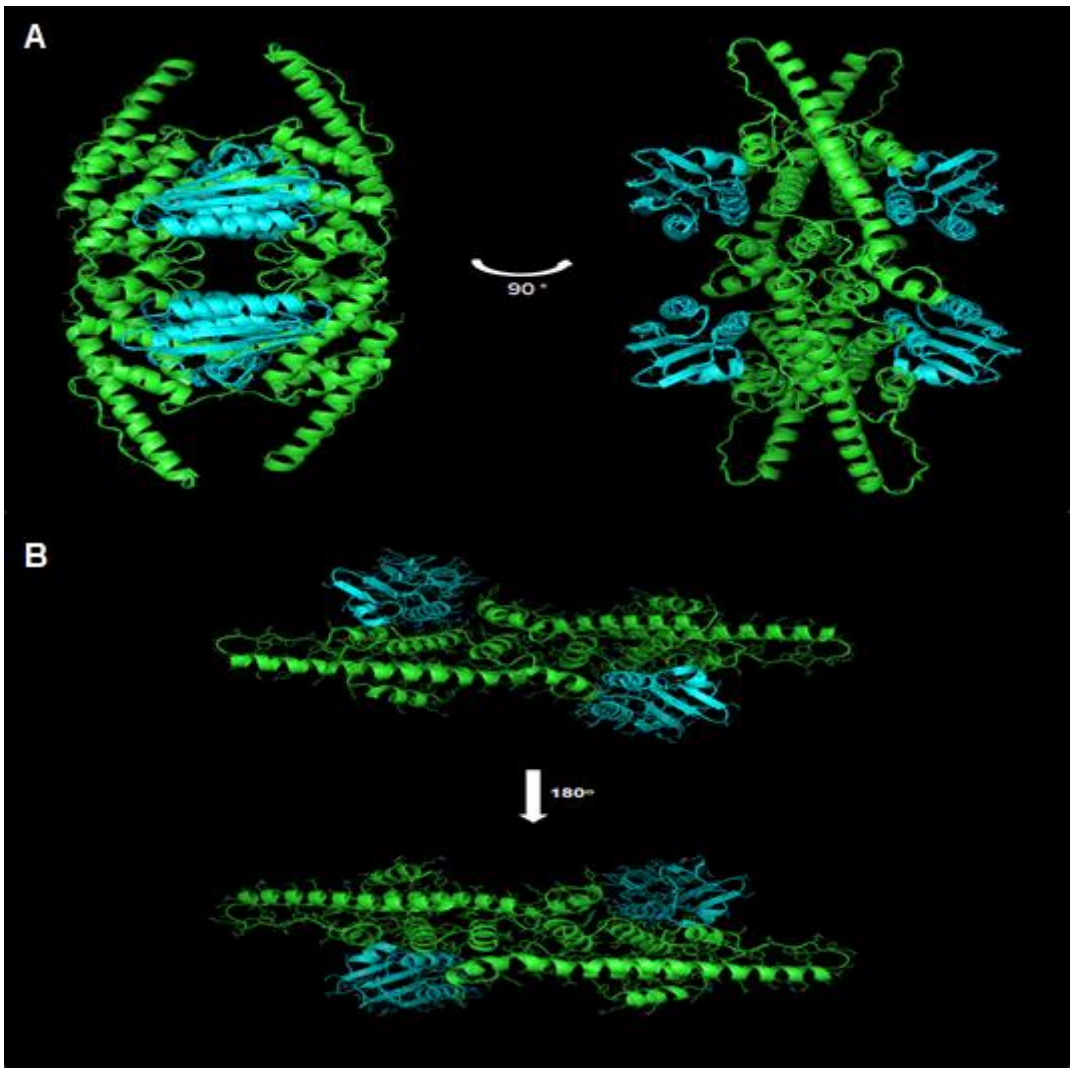

Figure 24. A crystal structure of the MtlR-HPr complex. (A) Ribbion diagrams showing two orthogonal views of the complex. MtlR (green) forms trapezoid shape tetramer and four monomer HPrs (cyan) are located at the outside of MtlR tetramer. (B) Crystal structure of heterotetrameric MtlR-HPr.

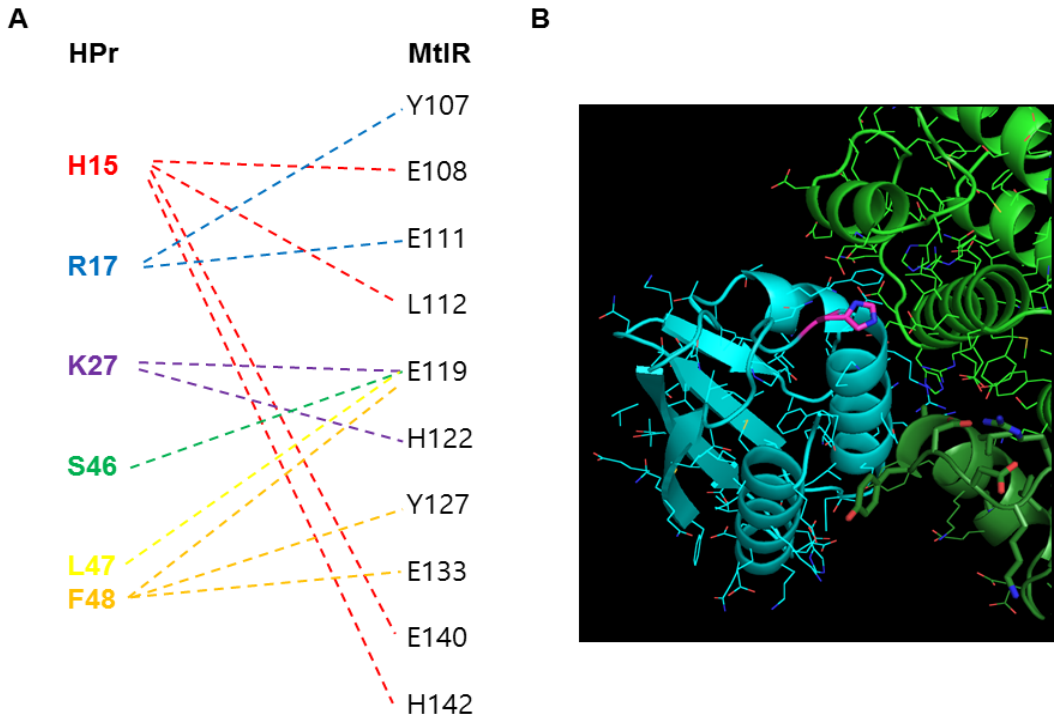

Figure 25. The binding interface between MtlR and HPr. (A) Schematic diagram denoting molecular interactions between MtlR and HPr. (B) Structure focusing on His15 of HPr. His15, a phosphorylation site of HPr is denoted by a purple color.

al., 2015) suggests that the binding interface of HPr is shared for the interaction with both MtlR and Rsd.

Chapter IV

Discussion

1. MtlR regulation by HPr.

Whereas the phosphorylation state of $EIIA^{Glc}$ is known to be crucial for the glucose repression of genes necessary for the transport and metabolism of non-PTS sugars in *E. coli* and many other Gram-negative bacteria, the phosphorylation state of HPr has been known as the central processing unit of CCR-related signal transduction in *B. subtilis* and many other Firmicutes (Görke & Stülke, 2008). While mechanisms of the preference between a PTS sugar and non-PTS sugars have been extensively studied (Deutscher *et al*., 2006), the mechanism for the preference among favored PTS sugars has barely been investigated in *E. coli*. In this study, I demonstrated that the preference between the two PTS sugars glucose and mannitol is independent of inducer exclusion and induction prevention by dephosphorylated $EIIA^{Glc}$ but determined solely by the phosphorylation state-dependent interaction of HPr with the mannitol operon repressor MtlR in *E. coli*. Dephosphorylated HPr, but not its phospho-form, specifically interacted with MtlR (Fig. 10), and the level of dephosphorylated HPr was significantly higher in the presence of glucose than in the presence of mannitol (Fig. 13). In the presence of mannitol alone, the expression of the *mtl* operon was remarkably induced (Fig. 5A). However, dephosphorylated HPr inhibited the induction of the *mtl* operon genes and thus the utilization of mannitol by binding to MtlR and enhancing its repressor activity even in the presence of mannitol (Figs. 14 and 17). Therefore, a complete loss of glucose preference over mannitol could be induced by *mtlR* deletion (Fig. 6B), additional expression of *mtlA* from a multi-copy plasmid (Fig. 7B), or substitution of

HPr with HPr(K27E), which still retains the phosphotransferase activity but is unable to interact with MtlR (Fig. 17B), all of which led to an increased expression level of the *mtl* operon even in the presence of glucose.

Taken together, all observations suggest that the preference of glucose over mannitol is entirely dependent on the prevention of the induction of the mannitol operon genes by the dephosphorylated HPr. It may be questioned, however, whether the difference between the dephosphorylation level of HPr in cells grown in glucose medium (99%) and that in mannitol-grown cells (64%) is large enough to support the selective inhibition of the induction of the mannitol operon genes by glucose but not by mannitol. To answer this question, I have compared the known difference in the phosphorylation state of $EIIA^{Glc}$ between cells in glucose medium and cells grown in lactose or melibiose medium since glucose preference over these less preferred sugars is known to be solely determined by the difference in the phosphorylation state of $EIIA^{Glc}$, and the induction of genes for the transport and metabolism of these less favored sugars is selectively inhibited by glucose in *E. coli*. Interestingly, while more than 95% of $EIIA^{Glc}$ was dephosphorylated in *E. coli* cells grown on glucose, $EIIA^{Glc}$ was approximately 60-70% dephosphorylated in cells grown on lactose and melibiose, respectively (Hogema *et al*., 1998). Because the difference in the dephosphorylation level of $EIIA^{Glc}$ between glucose- and lactose-grown cells is comparable to the difference in the dephosphorylation level of HPr between glucose- and mannitol-grown cells, the 35% difference in the dephosphorylation level of HPr is likely to be large enough to support glucose preference over mannitol.

2. HPr as a key player of sugar selectivity between glucose and mannitol.

In *E. coli* and other γ-proteobacteria, multiple roles of $EIIA^{Glc}$ have been identified, including regulation of adenylyl cyclase, the fermentation/respiration switch protein FrsA, glycerol kinase, and non-PTS transporters for lactose, maltose, and melibiose (Postma *et al*., 1993; Park *et al*., 2006; Koo *et al*., 2004). Considering the higher cellular concentration and smaller size than $EIIA^{Glc}$ (Rohwer *et al*., 2000), more regulatory functions have been anticipated for HPr in various Gram-negative bacteria. As a common PTS protein, HPr is highly conserved in Gram-positive and Gram-negative bacteria (Schnierow *et al*., 1989). HPr has long been recognized as the key player in CCR that regulates the activities of metabolic enzymes and transcriptional regulators in several Gram-positive bacteria (Görke & Stülke, 2008; Deutscher *et al*., 2014). As would be anticipated from the high similarity, increasing numbers of regulatory functions are being discovered for HPr in Gram-negative bacteria. HPr was first shown to regulate glycogen phosphorylase by direct interaction (Seok *et al*., 1997), and then activate the antiterminator BglG by phosphorylation (Görke & Rak, 1999) in *E. coli*. Recently, it was also shown that only unphosphorylated *E. coli* HPr formed a tight complex with Rsd and thus antagonized its anti-σ^{70} activity (Park *et al.*, 2013; Park *et al*., 2015). Furthermore, only unphosphorylated *Vibrio vulnificus* HPr was reported to interact with the C-terminal regulatory domain of pyruvate kinase A and stimulate its activity catalyzing the final step of glycolysis (Kim *et al*., 2015). It is interesting to note

that amino acid residues R17, K27, S46, L47, and F48 of HPr are involved in the phosphorylation-dependent interaction with both Rsd and MtlR in *E. coli* (Park *et al*., 2015) (Figs. 15 and 25). Therefore, these findings suggest the possibility that these residues might be commonly used for regulation of its target proteins by the phosphorylation-dependent protein-protein interactions in *E. coli*.

E. coli utilize mannitol as a one of primary carbon sources via the enzymes encoded by the *mtl* operon, whose regulatory mechanism has yet to be elucidated. Here in this study, I proposed that the *mtl* operon in *E. coli* is regulated through MtlR. Based on our data, three components are needed to maintain the repression effect on the *mtl* operon – MtlR, HPr and factor X (Fig. 26). Data shown in EMSA and the competition test (Figs. 19, 21 and 22) reveals that MtlR complex binds to the specific sites in the promoter region of the *mtl* operon nearby the CRP binding sites. Since the binding sites of MtlR and CRP are close but the regulation carried by MtlR and CRP is antagonistic, a competitive model between MtlR and CRP can be anticipated. MtlR shows stronger regulatory effect than CRP when HPr is overexpressed (Fig. 17B). Therefore, MtlR is thought to function as an anti-activator of cAMP-CRP complex in the regulation of the *mtl* operon.

In this study, I provide a novel mechanism for the CCR, which is independent of $EIIA^{Glc}$ but regulated by the phosphorylation state-dependent interaction with HPr. It would be interesting to show whether the inhibition of the induction of genes for the utilization of a less preferred PTS sugar by dephosphorylated HPr is specific for the preference between glucose and mannitol or widespread for the preference among PTS

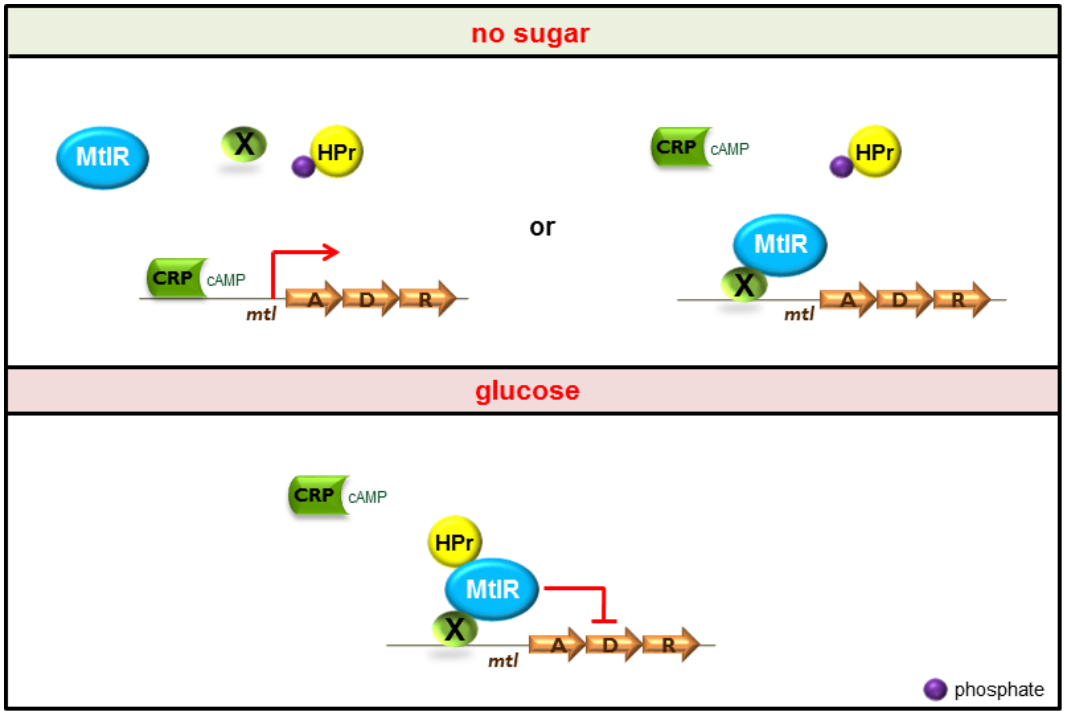

Figure 26. A proposed model for regulatory mechanism of MtlR modulated by HPr. Interaction between MtlR and *mtl* promoter is mediated by another DNA binding protein, factor X and MtlR-HPr-factor X complex tightly represses the expression of the *mtl* operon in the presenece of glucose.

sugars. As neither HPr nor MtlR possesses a DNA-binding domain, I need to identify a DNA-binding repressor involved in this regulation to fully understand the regulatory mechanism of the *mtl* operon by the HPr-MtlR complex.

References

Anderson J.W., Pullen K., Georges F., Klevit R.E., & Waygood E.B. (1993) The involvement of the arginine 17 residue in the active site of the histidine-containing protein, HPr, of the phosphoenolpyruvate:sugar phosphotransferase system of *Escherichia coli*. *J Biol Chem* 268(17):12325-12333.

Ann D.E. Fraser H.Y. (1982) Significance of beta-galactosidase repression in glucose inhibition of lactose utilization in *Escherichia coli*. *Current Microbiology* 7:241-244.

Bolivar F., Rodriguez R.L., Betlach M.C., & Boyer H.W. (1977) Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene* 2(2):75-93.

Chang A.C. & Cohen S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J bacteriol.* 134(3):1141-1156.

Chauvin F., Brand L., Roseman S. (1994) Sugar transport by the bacterial phosphotransferase system. Characterization of the *Escherichia coli* enzyme I monomer/dimer transition kinetics by fluorescence anisotropy. *J Biol Chem*. 269(32):20270-4.

Curtis S.J. & Epstein W. (1975) Phosphorylation of D-glucose in *Escherichia coli* mutants defective in glucosephosphotransferase,

mannosephosphotransferase, and glucokinase. *J Bacteriol* 122(3):1189-1199.

Datsenko K.A., Wanner B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*. 97(12):6640-5.

De Reuse H., Roy A., Danchin A. (1985) Analysis of the *ptsH-ptsI-crr* region in *Escherichia coli* K-12: nucleotide sequence of the *ptsH* gene. Gene. 35(1-2):199-207.

Deutscher J., Francke C., & Postma P.W. (2006) How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev.* 70(4):939-1031.

Deutscher J., Aké F.M., Derkaoui M., Zébré A.C., Cao T.N., Bouraoui H., Kentache T., Mokhtari A., Milohanic E., Joyet P. (2014) The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. *Microbiol Mol Biol Rev* 78(2):231-256.

Dörschug M., Frank R., Kalbitzer H.R., Hengstenberg W., Deutscher J. (1984) Phosphoenolpyruvate-dependent phosphorylation site in enzyme III^{glc} of the *Escherichia coli* phosphotransferase system. *Eur J Biochem*. 1;144(1):113-9.

Feucht B.U. & Saier M.H. Jr. (1980) Fine control of adenylate cyclase by the phosphoenolpyruvate:sugar phosphotransferase systems in *Escherichia coli* and *Salmonella typhimurium*. *J Bacteriol* 141(2):603-610.

Figge R.M., Ramseier T.M., & Saier M.H. Jr. (1994) The mannitol repressor (MtlR) of *Escherichia coli*. *J bacteriol.* 176(3):840-847.

Fox D.K., Meadow N.D., Roseman S. (1986) Phosphate transfer between acetate kinase and enzyme I of the bacterial phosphotransferase system. J Biol Chem. 15;261(29):13498-503.

García-Alles L.F., Zahn A., Erni B. (2002) Sugar recognition by the glucose and mannose permeases of *Escherichia coli*. Steady-state kinetics and inhibition studies. *Biochemistry*. 6;41(31):10077-86.

Görke B. & Rak B. (1999) Catabolite control of *Escherichia coli* regulatory protein BglG activity by antagonistically acting phosphorylations. *EMBO J* 18(12):3370-3379.

Görke B. & Stülke J. (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol* 6(8):613-624.

Herzberg O., Reddy P., Sutrina S., Saier M.H. Jr, Reizer J., Kapadia G. (1992) Structure of the histidine-containing phosphocarrier protein HPr from *Bacillus subtilis* at 2.0-A resolution. *Proc Natl Acad Sci U S A.* 15;89(6):2499-503.

Hogema B.M., Arents J.C., Bader R., Eijkemans K., Yoshida H., Takahashi H., Aiba H., Postma P.W.. (1998) Inducer exclusion in *Escherichia coli* by non-PTS substrates: the role of the PEP to pyruvate ratio in determining the phosphorylation state of enzyme IIA^{Glc}. *Mol Microbiol.* 30(3):487-498.

Hultquist D.E. (1968) The preparation and characterization of phosphorylated

derivatives of histidine. *Biochim Biophys Acta* 153(2):329-340.

Hurley J.H., Faber H.R., Worthylake D., Meadow N.D., Roseman S., Pettigrew D.W., Remington S.J. (1993) Structure of the regulatory complex of *Escherichia coli* III^{Glc} with glycerol kinase. *Science*. 259(5095):673-7.

Inada T., Kimata K., & Aiba H. (1996) Mechanism responsible for glucose-lactose diauxie in *Escherichia coli*: challenge to the cAMP model. *Genes Cells* 1(3):293-301.

Jeong J.-Y., Kim Y.-J., Cho N., Shin D., Nam T.-W., Ryu S., Seok Y.-J. (2004) Expression of *ptsG* encoding the major glucose transporter is regulated by ArcA in *Escherichia coli*. *J Biol Chem*. 279(37):38513-8.

Kim H.-J., Lee C.-R., Kim M., Peterkofsky A., & Seok Y.-J. (2011) Dephosphorylated NPr of the nitrogen PTS regulates lipid A biosynthesis by direct interaction with LpxD. *Biochem Biophys Res Commun* 409(3):556-561.

Kim H.-M., Park Y.-H., Yoon C.-K., & Seok Y.-J. (2015) Histidine phosphocarrier protein regulates pyruvate kinase A activity in response to glucose in *Vibrio vulnificus*. *Mol Microbiol* 96(2):293-305.

Kim Y.-J., *et al.* (2010) A mammalian insulysin homolog is regulated by enzyme IIA^{Glc} of the glucose transport system in *Vibrio vulnificus*. *FEBS Lett* 584(22):4537-4544.

Koo B.-M., Yoon M.-J., Lee C.-R., Nam T.-W., Choe Y.-J., Jaffe H.,

Peterkofsky A., Seok Y.-J. (2004) A novel fermentation/respiration switch protein regulated by enzyme IIA^{Glc} in *Escherichia coli*. *J bioll chem.* 279(30):31613-31621.

LaVallie E.R., DiBlasio E.A., Kovacic S., Grant K.L., Schendel P.F., McCoy J.M. (1993) A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Biotechnology* 11(2):187-193.

Lee C.A., Saier M.H. Jr. (1983) Mannitol-specific enzyme II of the bacterial phosphotransferase system. III. The nucleotide sequence of the permease gene. *J Biol Chem*. 258(17):10761-7.

Lee C.-R., Cho S.-H., Yoon M.-J., Peterkofsky A., & Seok Y.-J. (2007) *Escherichia coli* enzyme IIA^{Ntr} regulates the K^+ transporter TrkA. *Proc Natl Acad Sci U S A*. 104(10):4124-4129.

Lee C.-R., Kim M., Park Y.-H., Kim Y.-R., & Seok Y.-J. (2014) RppH-dependent pyrophosphohydrolysis of mRNAs is regulated by direct interaction with DapF in *Escherichia coli*. *Nucleic Acids Res* 42(20):12746-12757.

Lee S.J., Boos W., Bouche J.P., & Plumbridge J. (2000) Signal transduction between a membrane-bound transporter, PtsG, and a soluble transcription factor, Mlc, of *Escherichia coli*. *EMBO J* 19(20):5353-5361.

Lengeler J. & Lin E.C. (1972) Reversal of the mannitol-sorbitol diauxie in *Escherichia coli*. *J Bacteriol* 112(2):840-848.

Lengeler J. & Steinberger H. (1978) Analysis of regulatory mechanisms controlling the activity of the hexitol transport systems in *Escherichia coli* K12. *Mol Gen Genet* 167(1):75-82.

Lengeler J. & Steinberger H. (1978) Analysis of the regulatory mechanisms controlling the synthesis of the hexitol transport systems in *Escherichia coli* K12. *Mol Gen Genet* 164(2):163-169.

Lolkema J.S., Dijkstra D.S., ten Hoeve-Duurkens R.H., Robillard G.T. (1990) The membrane-bound domain of the phosphotransferase enzyme II^{mtl} of *Escherichia coli* constitutes a mannitol translocating unit. *Biochemistry*. 27;29(47):10659-63.

Loomis W.F., Jr. & Magasanik B. (1967) Glucose-lactose diauxie in *Escherichia coli*. *J bacteriol.* 93(4):1397-1401.

Lux R., Jahreis K., Bettenbrock K., Parkinson J.S., Lengeler J.W. (1995) Coupling the phosphotransferase system and the methyl-accepting chemotaxis protein-dependent chemotaxis signaling pathways of *Escherichia coli*. *Proc Natl Acad Sci U S A.* 92(25):11583-7.

Monod J. (1942) *Recherches sur la croissance des cultures bactériennes.*

Nam T.-W., *et al.* (2001) The *Escherichia coli* glucose transporter enzyme

IICB^{Glc} recruits the global repressor Mlc. *EMBO J* 20(3):491-498.

Napper S, *et al.* (1996) Mutation of serine-46 to aspartate in the histidine-containing protein of *Escherichia coli* mimics the inactivation by phosphorylation of serine-46 in HPrs from gram-positive bacteria. *Biochemistry* 35(35):11260-11267.

Nelson S.O., Scholte B.J., & Postma P.W. (1982) Phosphoenolpyruvate:sugar phosphotransferase system-mediated regulation of carbohydrate metabolism in *Salmonella typhimurium*. *J Bacteriol* 150(2):604-615.

Okada T., *et al.* (1981) Role of inducer exclusion in preferential utilization of glucose over melibiose in diauxic growth of *Escherichia coli*. *J Bacteriol* 146(3):1030-1037.

Osumi T. & Saier M.H. Jr. (1982) Regulation of lactose permease activity by the phosphoenolpyruvate:sugar phosphotransferase system: evidence for direct binding of the glucose-specific enzyme III to the lactose permease. *Proc Natl Acad Sci U S A.* 79(5):1457-1461.

Park Y.-H, Lee B.R., Seok Y.-J., Peterkofsky, A., (2006) *In vitro* reconstitution of catabolite repression in *Escherichia coli.* J Biol Chem. 281(10):6448-54.

Park Y.-H., Lee C.-R., Choe M., & Seok Y.-J. (2013) HPr antagonizes the anti-σ^{70} activity of Rsd in *Escherichia coli*. *Proc Natl Acad Sci U S A.* 110(52):21142-21147.

Pas H.H., ten Hoeve-Duurkens R.H., Robillard G.T. (1988) Bacterial phosphoenolpyruvate-dependent phosphotransferase system: mannitol-specific EII contains two phosphoryl binding sites per monomer and one high-affinity mannitol binding site per dimer. *Biochemistry*. 26;27(15):5520-5.

Postma P.W., Lengeler J.W., & Jacobson G.R. (1993) Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev.* 57(3):543-594.

Presper K.A., Wong C.Y., Liu L., Meadow N.D., Roseman S. (1989) Site-directed mutagenesis of the phosphocarrier protein. III^{Glc}, a major signal-transducing protein in *Escherichia coli*. *Proc Natl Acad Sci U S A.* 86(11):4052-5.

Reddy P., Peterkofsky A., & McKenney K. (1989) Hyperexpression and purification of *Escherichia coli* adenylate cyclase using a vector designed for expression of lethal gene products. *Nucleic Acids Res.* 17(24):10473-10488

Reizer J., *et al.* (1992) Functional interactions between proteins of the phosphoenolpyruvate:sugar phosphotransferase systems of *Bacillus subtilis* and *Escherichia coli*. *J Biol Chem.* 267(13):9158-9169.

Rohwer J.M., Meadow N.D., Roseman S., Westerhoff H.V., & Postma P.W. (2000) Understanding glucose transport by the bacterial

phosphoenolpyruvate:glycose phosphotransferase system on the basis of kinetic measurements *in vitro*. *J Biol Chem.* 275(45):34909-34921.

Roy, A., Danchin, A., Joseph, E., and Ullmann, A. (1983) Two functional domains in adenylate cyclase of *Escherichia coli*. *J Mol Biol*. 165, 197-202.

Satishchandran C. & Boyle S.M. (1984) Antagonistic transcriptional regulation of the putrescine biosynthetic enzyme agmatine ureohydrolase by cyclic AMP and agmatine in *Escherichia coli*. *J Bacteriol.* 157(2):552-559.

Schnierow B.J., Yamada M., & Saier M.H. Jr. (1989) Partial nucleotide sequence of the *pts* operon in *Salmonella typhimurium*: comparative analyses in five bacterial genera. *Mol Microbiol.* 3(1):113-118.

Seok, Y.-J., Lee B.R., Zhu P.P., and Peterkofsky, A. (1996). Importance of the carboxyl-terminal domain of enzyme I of the *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase system for phosphoryl donor specificity. *Proc Natl Acad Sci U S A.* 93, 347-351.

Seok Y.-J., *et al.* (1997) High affinity binding and allosteric regulation of Escherichia coli glycogen phosphorylase by the histidine phosphocarrier protein, HPr. *J Biol Chem.* 272(42):26511-26521.

Seok Y.-J., Zhu P.P., Koo B.-M., Peterkofsky A. (1998) Autophosphorylation of enzyme I of the *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase system requires dimerization. *Biochem Biophys Res*

Commun. 1998 Sep 18;250(2):381-4.

Seok Y.-J., Koo B.-M., Sondej M., Peterkofsky A. (2001) Regulation of *E. coli* glycogen phosphorylase activity by HPr. *J Mol Microbiol Biotechnol*. 3(3):385-93.

Shi X., Karkut T., Alting-Mees M., Chamankhah M., Hemmingsen S.M., Hegedus D.D. (2003) Enhancing *Escherichia coli* electrotransformation competency by invoking physiological adaptations to stress and modifying membrane integrity. *Anal Biochem*. 320(1):152-5.

Siebold C., Flükiger K., Beutler R., Erni B. (2001) Carbohydrate transporters of the bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS). *FEBS Lett*. 504(3):104-11.

Stock J.B., Waygood E.B., Meadow N.D., Postma P.W., & Roseman S. (1982) Sugar transport by the bacterial phosphotransferase system. The glucose receptors of the *Salmonella typhimurium* phosphotransferase system. *J Biol Chem* 257(23):14543-14552.

Tan K., Clancy S., Borovilos M., Zhou M., Hörer S., Moy S., Volkart L.L., Sassoon J., Baumann U., Joachimiak A. (2009) The mannitol operon repressor MtlR belongs to a new class of transcription regulators in bacteria. *J Biol Chem*. 25;284(52):36670-9. doi: 10.1074/jbc.M109.062679.

Tchieu J.H., Norris V., Edwards J.S., Saier M.H. Jr. (2001) The complete

phosphotransferase system in *Escherichia coli*. *J Mol Microbiol Biotechnol*. 3(3):329-46.

van Weeghel R.P., Meyer G., Pas H.H., Keck W., Robillard G.T. (1991) Cytoplasmic phosphorylating domain of the mannitol-specific transport protein of the phosphoenolpyruvate-dependent phosphotransferase system in *Escherichia coli*: overexpression, purification, and functional complementation with the mannitol binding domain. *Biochemistry* **30:** 9478-9485

Wittekind M., Reizer J., Klevit R.E. (1990) Sequence-specific 1H NMR resonance assignments of *Bacillus subtilis* HPr: use of spectra obtained from mutants to resolve spectral overlap. *Biochemistry*. 7;29(31):7191-200.

Yang, J.K., and Epstein, W. (1983) Purification and characterization of adenylate cyclase from *Escherichia coli* K12. *J Biol Chem*. 258, 3750-3758.

Zheng D., Constantinidou D., Hobman J.L., Minchin S.D. (2004) Identification of the CRP regulon using *in vitro* and *in vivo* transcriptional profiling. *Nucleic Acids Res*. 32(19):5874-93.

국문 초록

박테리아의 phosphoenolpyruvate: sugar phosphotransferase system (PTS)은 여러 종류의 당을 수송하는 것과 동시에 이 당들을 인산화시키는 기능을 담당한다. PTS는 모든 당들에 공통으로 사용되는 2가지 세포질 단백질인 EI, HPr과 당특이적으로 사용되는 EII 단백질로 구성된다. EII 단백질은 일반적으로 A, B, 그리고 C라는 3가지 domain으로 구성되어 있으며 이 중 C domain이 세포막에 존재하면서 실질적으로 당의 수송에 관여한다. PTS 단백질은 당을 수송하는 기능 이외에도 다른 단백질과의 상호작용을 통해 세포 내 신호 전달이나 대사 조절에도 관여하는 것으로 알려져 있다.

선택적 당 수송은 미생물에서 순차적 당 수송을 가능하게 하는 현상으로 그 분자적 기작은 대장균과 바실러스균에서 많이 연구가 되었다. 포도당은 이 균주들에서 가장 선호되는 당이며 PTS를 통해 수송된다. 선택적 당 수송을 설명하기 위해 이전까지의 연구들을 통해 inducer exclusion과 induction prevention 이라는 2가지 기작에 기반한 모델이 제시되었다. Inducer exclusion이란 선호당이 존재할 때 직접적으로 비선호당의 수송을 억제하는 현상을 의미하며 induction prevention이란 adenylate cyclase의 활성을 억제하는 현상을 의미한다. 이 두 가지 분자 기작은 모두 $EIIA^{Glc}$의 인산화 상태에 의해 조절받는다. 가장 대표적으로 알려져 있는 포도당과 젖당 사이의

선호도는 실제로 $EIIA^{Glc}$의 인산화 상태에 의해 조절된다. 하지만 다른 여러 종류의 PTS 당들 사이의 선호도는 연구된 바가 많이 없으며 포도당과 만니톨 사이의 당선호도는 포도당-젖당의 분자 매커니즘과는 전혀 다르게 조절되는 것으로 생각된다.

본 연구에서는 대장균에서 두 가지 PTS 당인 포도당과 만니톨 사이의 분자 조절 기작에 대해 연구를 수행하였다. 놀랍게도 현재의 모델에서 중요하다고 알려져 있는 $EIIA^{Glc}$ 결손 균주에서는 야생형 균주와 마찬가지로 포도당을 선호하지만 만니톨 오페론 전사 조절자인 MtlR이 결손된 균주에서는 두 당을 동시에 사용하는 현상을 확인하였다. MtlR은 PTS 구성 단백질인 HPr이 탈인산화되었을 때 특이적인 상호작용을 하며 MtlR-HPr 복합체는 만니톨 오페론의 발현을 억제하여 만니톨의 사용을 억제하는 것으로 관찰되었다. 여러가지 추가 실험을 통하여 MtlR이 만니톨 오페론의 프로모터 부분에 작용한다는 사실을 확인하였으며 결정화 실험을 통해 실제로 MtlR과 HPr 복합체의 구조를 규명하였다.

이 논문에서는 포도당과 만니톨 사이의 당 선호도 조절 기작에, 기존의 모델에서 중요하다고 여겨지던 $EIIA^{Glc}$가 아닌 HPr이 중요한 역할을 한다는 것을 보였다.

주요어 :

대장균, 선택적 당수송, PTS, HPr, MtlR

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